BARD- Final Scientific Report 2012

Cover Page

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Project Title: The role of cysteine partitioning into glutathione and methionine synthesis during normal and stress conditions

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
flux analysis, substrate competition, oxidative stress, metabolic profiling

Abbreviations commonly used in the report, in alphabetical order**:** CGS, cysthathionine gamma-synthase; Cys, cysteine; GSH, glutathione; Met, methionine; SAM, S-adenosyl methionine

Budget: IS: $ US: $ Total: $

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged				
Submitted, in review, in preparation	2 (in preparation)	0	1 (submission) 2 (in preparation)	5
Invited review papers		0	2	2
Book chapters		0		0
Books		0		0
Master theses		0		0
Ph.D. theses		0		0
Abstracts		0	3	3
Not refereed (proceedings, reports, etc.)		0	0	0

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Yaron Dekel "The role of cysteine partitioning into glutathione and methionine synthesis during normal and stress conditions.

Dr. Shy Kosevitzki "The role of methionine recycles pathways during abiotic stresses"

Dr. Yael Hacham "The role of glutathione on the expression level of genes in Arabidopsis plants"

Dr. Boris Eyheraguibel (full time) Metabolic profiling of plants under oxidative stress

Dr. Jung Moon Yoon (half time) flux analysis

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings				0
Longer Visits (Sabbaticals)				0

I (Rachel Amir) must write some personally comments concerning the visiting and meeting and the publications writing. In end of May 2011 the doctors found in my older son Ewing sarcoma. Since then I found myself for long time in the hospitals with my son. So although David intends to visit me at summer of 2011, and I intend to visit his laboratory in 2012, I cancel these meetings, because of the unexpected situation. This is also the reason why the publications delay. The situation is now better, and David with me writing our common publications. We still working to finish some of the analyses, but I expected that we will submit in a few months the first of our publication, and soon after it the second one.

Description Cooperation: Both PI were communicated quit often by emails and by Skype and methods as well as results were transfer between our laboratories.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				0
Licensed				0

Publications (Attached)

Amir R, Han T, Ma F. (2012) Bioengineering approaches to improve the nutritional values of seeds by increasing their methionine content. Molecular Breeding 29: 915-924.

Galili G, Amir R. (2012) Fortifying plants with the essential amino acids lysine and methionine to improve nutritional quality. Plant Biotechnol. J. (In press).

Abstracts in conferences

Dekel Y, Matityahu I, Hacham Y, Amir R (2011) The competition between glutathione and methionine for cystein: glutathione is down regulating the protein levels of cystathionine γ synthase. The 6th Congress of the Federation of the Israeli Society of Experimental Biology. February 7-10, 2011 Eilat, Israel

Matityahu I, Godo I, Hacham Y, Avzach A, Amir R (2012) Enhancing methionine accumulation in tobacco seeds reveals a physiological link between methionine and glutathione and its impact on seed germination. Conference on Plant Metabolism. Banff, Alberta, Canada, June 28 – July 2.

Hacham Y, Matityahu I, Dekel Y, Amir R (2012) The role of cysteine partitioning into glutathione and methionine under normal and stress conditions. Plant Biology Congress jointly organized by FESPB and EPSO. Freiburg, Germany. July 28- 3 August.

The potential publications, putative names and their putative contents:

1. The competition between glutathione and methionine for their common substrate, cysteine-this will contains our findings, that the Flux towards methionine synthesis is relatively high, and thus the methionine synthesis competes with GSH synthesis for cysteine.

2. Glutathione is down regulating the protein levels of cystathionine γ synthase- will described the findings showing that GSH regulates the protein level of CGS

3. Glutathione affects the transcript level of genes involved in stresses- will described the results obtained from the microarray analyses

4. Enhancing methionine accumulation in tobacco seeds reveals a physiological link between methionine and glutathione and its impact on seed germination- will described the results obtained in transgenic seeds expressing CGS and have higher levels of methionine

5. Light and dark regulates the level of cystathionine γ synthase –described the effects of light on methionine, GSH and cysteine levels (submitted).

The role of GSH on methionine synthesis

The objective of this research is to study the nature of the competition for cysteine (Cys), the first organic sulfur-containing compound, between its two main metabolites, glutathione (GSH), which plays a central role in protecting plants during various stresses, and methionine (Met), an essential amino acid, which through its metabolite *S*-adenosyl-Met, regulates essential processes and metabolites in plant cells.

Our results demonstrate that during unstressed conditions the level of GSH remained quit stable (about 500 nmol/g fresh weight), and also the level of Met (about 30 nmol/g fresh weight). The level of GSH is usually more than one-order fold than of Met. However during oxidative stress, the level of GSH significantly increases (about 2500 nmol/g fresh weight), while that of Met is significantly reduced (about 10 nmol/g fresh weight). The results further suggest that GSH down regulates the expression level of the first unique enzyme of Met synthesis, cystathionine γ-synthase (CGS), thus reducing the Met content. This reduction leaves more Cys that can be channelled towards GSH synthesis under stress conditions. The low Met content most probably affects plant metabolism and plant growth.

We reveal the role of GSH on the expression level of CGS and on the content of Met. Therefore, using Arabidopsis as a model plant and by conducting feeding experiments we tested how transiently altered levels of GSH affect CGS and the levels of Met. The Arabidopsis plants were grown in liquid media of B5 in a rotary shaker and the tested agents were being directly administered into the culture medium. First, we calibrated the time span from application of the agents up to experiment termination. Secondly, we set the concentrations of GSH; BSO (a specific inhibitor of ECS); and Dithiothreitol (DTT) that is used as a control for GSH since it has the ability to act as thiol-disulfide modifier. It was found that plants s H reduced the expression level of CGS at the protein gher levels of CGS. DTT had no effect (Fig. 1). Since the level of CGS mRNA wasn't altered, these results suggest that GSH negatively modulates the protein expression level of AtCGS.

Figure 1- Protein expression level of Arabidopsis CGS after treatment with: A. Methionine; B. DTT; and C. BSO. Control plans were given PBS. Lower panel – Poncue images.

Arabidopsis plants that were grown in liquid media showed a depressed phenotype, and thus we decided to replace the B5 system. To this end, the Arabidopsis plants were grown on square agar plates that stand vertically. GSH, BSO and DTT were added to the roots of 19-days old plants and the GSH content were measured in the leaves of these plants. The GSH content increased 14 fold in GSH treated plants compared to plants treated with water. Since GSH application lead to higher cysteine levels we next examined its level. It was found that cysteine increased 3.4 times above the level found in control plants, suggesting that GSH penetrates the roots and reaches the shoots. This system gave similar results to the plants growing in the B5 liquid media, though the plants grow better, and therefore our further studies will be preformed with this system.

This modulation of GSH could be preformed either by affecting the translation rate or affecting the protein stability in a post translation modification. To distinguish between these two mechanisms, we used the *in vitro* coupled transcription/ translation system. cDNA encoding AtCGS were transcribed/translated in the presence of GSH at different concentration and with DTT and Met that were used as controls. To further study the role of GSH on the translation process, we used the *in vitro* translation system. The results showed that GSH reduced the transcript expression levels of the control luciferase gene, and thus the reliability of both *in vitro* systems was reduced. This system will not be used for further analysis.

To further study if GSH influences the polysomes level of CGS and thus affect the translation rate, polysomes experiments were preformed (Fig. 2). While the first experiments suggests that GSH control the translation rate of CGS, many other experiments that we have done this year lead us to conclude that basically, GSH does affect the translation rate of CGS. This further suggests that GSH affect CGS on the post-translational level.

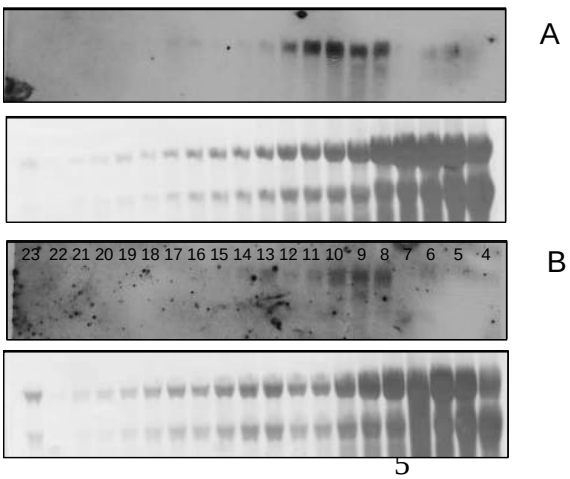

Figure 2- Polysome analysis of CGS protein levels after 6 hours treatment with PBS (A) or 2.5 mM GSH (B). The lower panel is the methylene blue image.

To revels this point, we next studied if GSH reduced the stability of CGS by post translation modification. To this end, the Arabidopsis plants were grown on agar at square Petri dishes for 19 days supplemented with GSH, cyclohexamide, a translation inhibitor, and both GSH/ cyclohexamide. If GSH affects the stability of CGS post translationaly, we expected to find that the combinations of the GSH/ cyclohexamide will lead to lower levels of CGS compared to cyclohexamide or GSH alone. The results (Fig. 3) showed indeed that as we expected, the expression level of CGS reduced when these two compounds were added together. The expression levels of the other proteins such Actin and T-APX were not significantly alter. This suggests that GSH affects CGS on the post translational level.

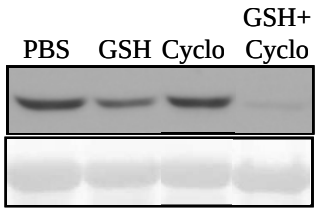

Figure 3- Protein expression level of Arabidopsis CGS after treatment with: PBS used as a control; GSH; cyclohexamide and combination of GSH and cyclohexamide.

Elucidation the regulatory role of GSH on AtCGS levels

The results obtained above suggest that GSH regulates the level of AtCGS in a post-translation manner. Previously we have found that the N-terminal region of AtCGS play significant role in regulated the transcript level of AtCGS. Thus we studied if transgenic tobacco plants overexpressing the AtCGS without its N-terminal region are sensitive to GSH. The results are similar to plants overexpressing the full-length AtCGS. Thus, the N-terminal region is not take play role in this regulation, suggesting that the regulation occurs on the region which it's quite conserved among plants that contain the catalytic domain.

Next we try to gain more knowledge on the mechanism in which such regulation occurs. Proteins can be modified post-transnationally by the attachment of a GSH molecule to cysteine residues by disulfide bonds (glutathionylation). Using bioinformatics tools we have identified a putative glutathionylation site in CGS. AtCGS protein without its transient-peptide (Full-length CGS), contain 9 cysteine residues, only one of them is located within the

N- terminal region of the protein (Fig 4a). In our lab we have previously expressed the *Arabidopsis* CGS without its N-terminal region (truncated CGS, T-CGS) in Bactria and tobacco plants. This form of CGS was active in both systems (Hacham 2002). Using bioinformatics tools we have identified a putative glutathionylation site in the T-CGS protein. To test the possibility that CGS undergoes glutathionylation we expressed the T-CGS with 6xHIS in *E.coli*. Purification of His tagged CGS was achieved by nickel-column under nondenaturing conditions (Fig. 4b). The purified protein will be farther tested for glutathionylation sites by several methods including: detection of bound GSH by anti-GSH antibodies and mass spectrometric analysis according to Van der linde (Van der linde et al, 2011). In addition we also studied the possibility that CGS undergoes glutathionylation is being tested in collaboration with Dr. Nicolase Rouhier (Institute Universities de France). However, we did not get yet the results.

(A)

VRQLSIKARRNCSNIGVAQIVAAKWSNNPSSALPSAAAAAATSSASAVSSAASAAAASSAAAAPVAAAPPVVLKSVDEEVVVA
EEGIREKIGSVQLTDSKHSFLSSDGSLTVHAGERLGRGIVTDAITTPVVNTSAYFFKKTAELIDFKEKRSVSFEYGRYGNPTT
VVLEDKISALEGAESTLVMASGMCASTVMLLALVPAGGHIVTTTDCYRKTRIFMENFLPKLGITVTVIDPADIAGLEAAVNEF
KVSLFFTESPTNPFLRCVDIELVSKICHKRGTLVCIDGTFATPLNQKALALGADLVVHSATKYIGGHNDVLAGCICGSLKLVS
EIRNLHHVLGGTLNPNAAYLIIRGMKTLHLRVQQQNSTAFRMAEILEAHPKVSHVYYPGLPSHPEHELAKRQMTGFGGVVSFE
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(B)

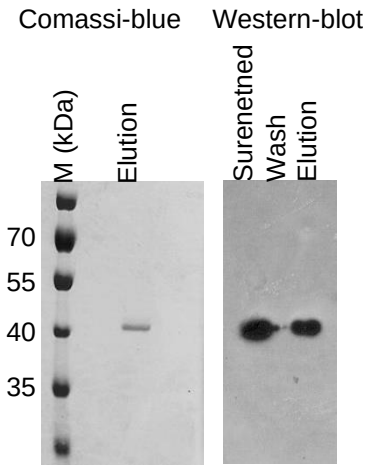


Fig 4 - (A) Protein sequence of *Arabidopsis* CGS without the transient-peptide (full-length CGS). The beginning of truncated from of CGS are marked in yellow. The cysteine residues are marked in green.
(B) CGS purification by nickel-column.

Elucidating the regulatory role of Met on the level of ECS

The first enzyme of GSH synthesis, γ-glutamylcysteine synthetase (γ-ECS), competes with CGS for cysteine as their common substrate. The results described above suggest that GSH affect the level of AtCGS, but we also study the possibility that ECS levels are affected by high Met levels. To this end we have measured the transcript level of ECS following Met or GSH addition. The results show (Figure 5C) that high Met level slightly positive affects the transcript level of ECS. However, the protein level of ECS does not significantly alter by application of Met.

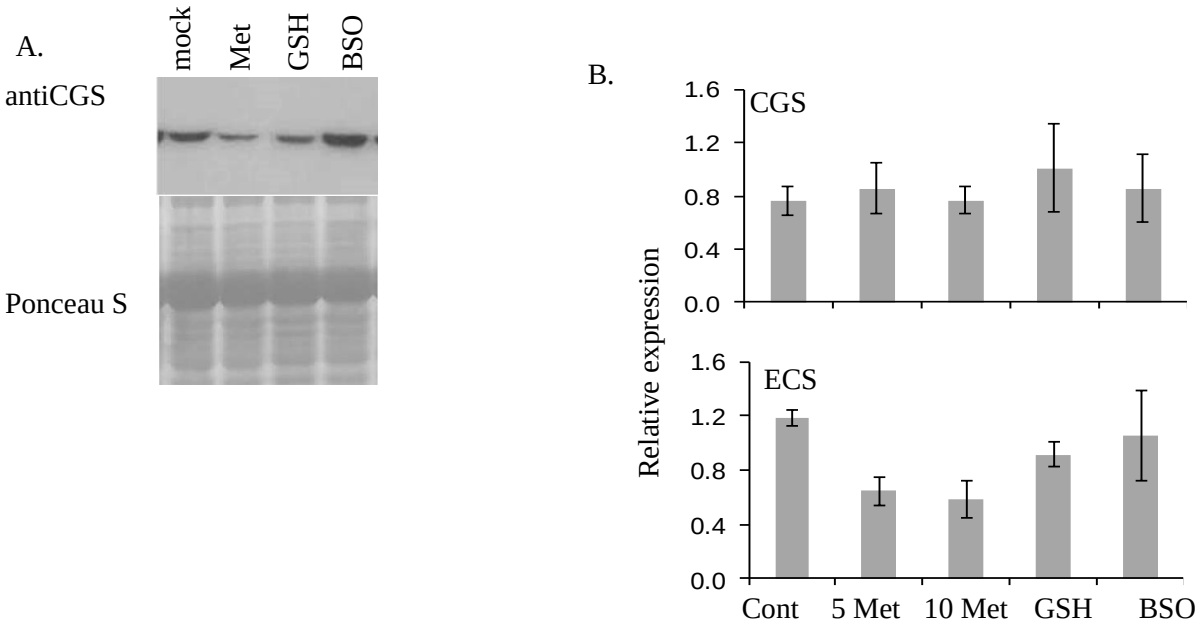


Figure 5. A. Protein expression level of Arabidopsis CGS after treatment with: MS treatment (Mock), 5 mM Methionine (Met), 5 mM Glutathione (GSH) and 2 mM BSO; B. Quantitative Real Time- PCR showing CGS and ECS transcript level. The data represent three independent experiments each with three biological repeats.

Elucidation the role of GSH on gene expression (with Prof. Gad Galili)

In order to study the possible effects of elevated GSH levels on gene expression profile, we conducted a transcriptome analysis of *Arabidopsis* plants that were treated with GSH. Our hypothesis was that high levels of GSH present during various stresses can serve as signals to regulate gene expression levels. To address this, Arabidopsis plants grown vertically on square agar plates were treated for 4 hours with 5 mM GSH or MS as a control. Total RNA extracted from these plants was taken for microarray analysis using Affymetrix AtH1 chips. The microarray results indicated that the expression of 453 genes showed a

significant change (p value<0.05) of at least 1.5-fold between the control and GSH treatments. Expression of 261 genes was up regulated and expression of 192 genes was down regulated (Fig. 1).

Figure 1 - Classification of genes that were significantly up regulated (A) or down regulated (B) by at least 1.5-fold between the control and GSH treatments.

Genes that significantly changed by 1.5 fold were subjected to a PageMan over-representation (enrichment) analysis in order to elucidate the biological processes associated with them. The major biological processes enriched among the genes that were significantly up-regulated in GSH treatment compared to the control, were mainly associated with: Photosynthesis, Starch degradation, Hormone metabolism (especially genes associated with jasmonate), Biotic stress (especially genes associated with PR-proteins), cytochrome P450 genes, regulation of

transcription, Signaling (especially genes associated with receptor kinases and calcium) (Table 1). The major biological processes associated with the genes that were significantly down regulated in GSH treatment compared to the control, were mainly associated with: Hormone metabolism (especially genes associated with auxin), genes associated with abiotic stress and with development. In addition GSH treatment caused a decrease in the expression of different peroxidases and cell wall proteins (Table 2).

Table 1. Over-representation (enrichment) analysis of genes that were up-regulated by GSH*. Functional categories that are over-represented in the list of genes that exhibit significant changes in expression levels between control and GSH treatments ($p<0.05$ ratio>3).

BIN	Binname	Count	p value	Ratio
1	Photosynthesis	6	2.118E-02	2.8
2	Major CHO metabolism	4	2.733E-02	3.5
2.2	Major CHO metabolism, degradation	3	3.728E-02	4.1
2.2.2	Major CHO metabolism, degradation, starch	3	3.194E-03	10.1
2.2.2.1	Major CHO metabolism, degradation, starch, starch cleavage	3	3.923E-04	20.1
11.3	Lipid metabolism, Phospholipid synthesis	3	2.529E-02	4.8
11.9	Lipid metabolism, lipid degradation	4	4.842E-02	2.9
17	Hormone metabolism	11	2.459E-02	2.0
17.7	Hormone metabolism, jasmonate	3	6.320E-03	7.9
17.7.1	Hormone metabolism, jasmonate, synthesis-degradation	3	1.263E-03	13.8
19	Tetrapyrrole synthesis	3	1.486E-02	5.8
20	Stress	26	2.912E-06	2.8
20.1	Stress, biotic	22	6.717E-09	4.4
20.1.7	Stress, biotic, PR-proteins	11	2.428E-05	4.7
26	Misc	23	4.332E-02	1.5
26.1	Misc, cytochrome P450	6	2.765E-02	2.6
26.8	Misc*, nitrilases, nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	4	9.025E-03	4.9
27	RNA	14	2.561E-03	0.5
27.3	RNA, regulation of transcription	13	3.448E-02	0.6
28	DNA	4	2.849E-02	0.4
29	Protein	24	2.992E-02	0.7
30	Signaling	27	6.409E-04	2.0
30.2	Signaling, receptor kinases	13	2.778E-03	2.5
30.2.17	Signaling, receptor kinases, DUF 26	3	1.399E-02	5.9
30.3	Signaling, calcium	8	3.307E-03	3.3
35	Not assigned	69	1.459E-02	0.8
35.2	Not assigned, unknown	48	4.769E-02	0.8

* Aanalysis was performed by PageMan. Only functional categories with more than two genes are shown. CHO, carbohydrates; Misc, large enzymes families that cannot be attributed to any pathway.

Table 2. Over-representation (enrichment) analysis of genes that were down-regulated by GSH *. Functional categories that are overrepresented in the list of genes that exhibit significant changes in expression levels between mock and GSH treatments. $p<0.05$ ratio>3.

BIN	Binname	Count	p value	Ratio
10.5	Cell wall, cell wall proteins	3	3.225E-02	4.3
11.9.3	Lipid metabolism, lipid degradation, lysophospholipases	3	6.885E-03	7.7
16.2	Secondary metabolism, phenylpropanoids	3	3.028E-02	4.4
16.2.1	Secondary metabolism, phenylpropanoids, lignin biosynthesis	3	3.444E-03	9.9
16.8	Secondary metabolism, flavonoids	3	3.225E-02	4.3
17	Hormone metabolism	10	6.807E-03	2.5
17.2	Hormone metabolism, auxin	6	1.873E-03	4.7
17.2.3	Hormone metabolism, auxin.induced-regulated-responsive-activated	5	3.543E-03	4.9
20	Stress	13	2.874E-02	1.9
20.2	Stress, abiotic	7	4.243E-02	2.2
20.2.99	Stress, abiotic, unspecified	5	1.674E-03	5.9
26	Misc	24	4.303E-04	2.2
26.12	Misc, peroxidases	9	4.407E-09	16.2
26.21	Misc, protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	3	1.911E-02	5.3
27.3.5	RNA, regulation of transcription, ARR	3	9.259E-04	15.5
29.5.3	Protein, degradation, cysteine protease	4	6.351E-03	5.4
33	Development	11	2.807E-02	2.0
33.99	Development, unspecified	11	1.814E-02	2.2
35	Not assigned	47	7.117E-03	0.7
35.1.40	Not assigned, no ontology, glycine rich proteins	3	2.478E-02	4.8
35.1.41	Not assigned, no ontology, hydroxyproline rich proteins	3	1.241E-02	6.2
35.2	Not assigned, unknown	23	5.454E-05	0.5

* Aanalysis was performed by PageMan. Only functional categories with more than two genes are shown. Misc, large enzymes families that cannot be attributed to any pathway.

GSH has been reported to play an important role in plant responses during biotic stresses. Low content of GSH in *pad2-1* mutant confers an enhanced susceptibility to various fungal, bacterial and omycete pathogens as well as insect herbivores (Dubreuil-Maurizi and Poinssot Plant Signaling & Behavior, Volume 7 Issue 2). However our knowledge of GSH-related molecular mechanisms underlying plant defense responses still remains limited.

Recently it was reported that transgenic tobacco with enhanced level of GSH had enhanced expression of genes belong to NPR1-dependent SA-mediated pathway (Chattopadhyay 2011)

In our microarray results at GSH treatment there was a preferential change in transcripts coding for proteins that were associated with stress. The expression of PR1 and PR5 (pathogenesis-related) proteins, increased by 2.46 and 2.1 fold (respectively). It was previously reported that the level of GSH but not its redox state participates in the promotion of PR-1 gene expression (Senda and Ogawa, 2004). In addition the expression of genes associated with Jasmonic acid synthesis and signaling: LOX2, LOX3, JAZ1, JAZ8 were up regulated.

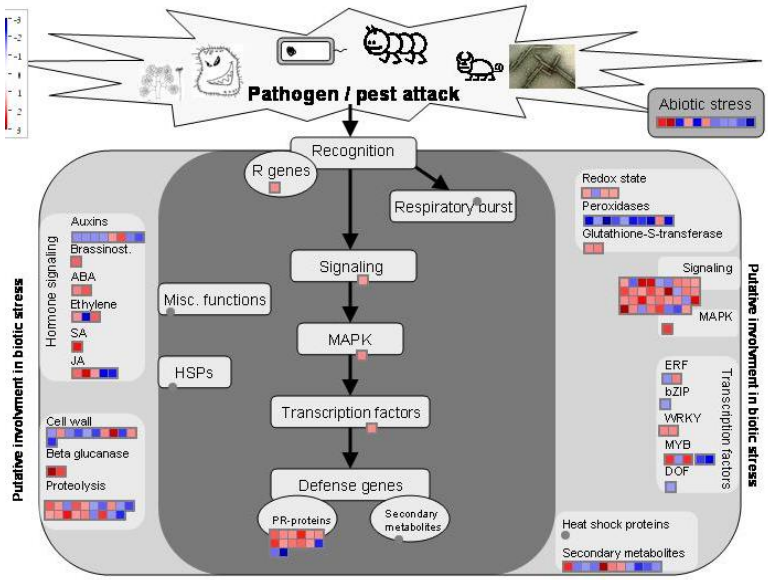
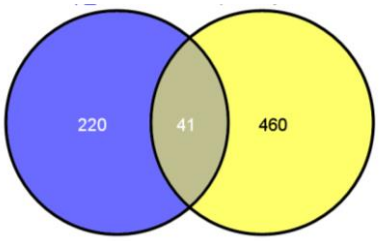


Figure 2. MapMan overview of genes related to biotic stress that significantly changed in response to GSH treatment.

To elucidate the influence of elevated levels of GSH on genes that response to biotic stress, we utilized previously published microarray data of Arabidopsis plants inoculated with necrotrophic fungal pathogen "*Botrytis cinerea*" (Ferrari et al., 2007). Venn-diagram analysis showed that 41 genes were commonly induced, and 5 genes were commonly repressed. The major biological processes associated with 41 up-regulated genes, (15.7% from genes that were significantly up regulated in GSH treatment) included genes that associated to jasmonate metabolism, biotic stress and nitrilases (Table 3).

(A)



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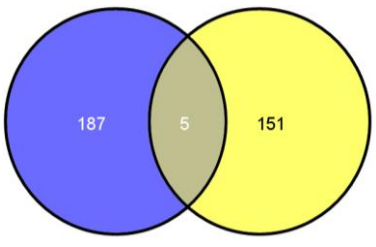


Figure 3. Venn diagrams illustrating the number of genes that were up regulated (A) or down regulated (B) by GSH treatment (blue) and biotic stress caused during *B. cinerea* infection (yellow).

Table 3. Enriched biological process associated with the genes that are up regulated in both GSH treatment and the *B. cinerea* treated plants.

BIN	Binname	Count	p value	Ratio
17.7	Hormone metabolism, jasmonate	2	1.616E-03	33.6
20.1	Stress.biotic	3	4.286E-02	3.8
26.8	Misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	3	2.853E-04	23.4

Next we compared our microarray data with microarray of *Arabidopsis* plants treated with salicylic acid analog (BTH). Venn-diagram analysis showed that 90 genes (34.5% from genes that were significantly up regulated in GSH treatment) were commonly induced and 23 genes were commonly repressed. Among the major biological processes that were up-regulated are genes associated with biotic stress, cytochrome P450 and signalling by receptor kinases.

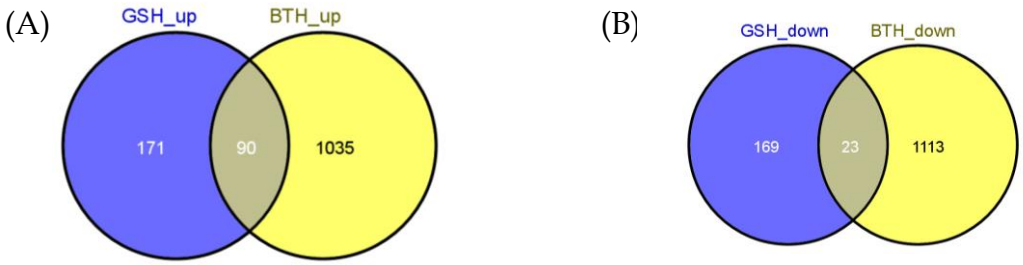
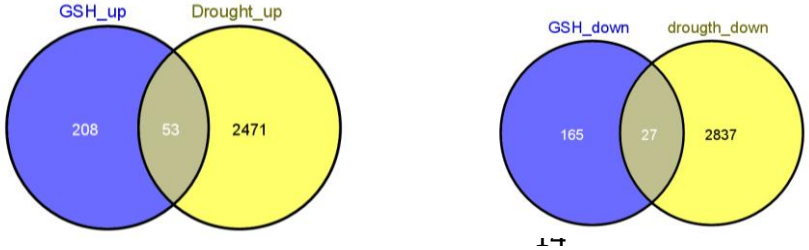


Figure 4. Venn diagrams illustrating the number of genes that were up regulated (A) or down regulated (B) by GSH treatment (blue) and treatment with BTH (yellow).

In addition we checked the shared effect of GSH and abiotic stress on gene expression by comparing our microarray data with publication describing microarrays after different abiotic stress including drought, NaCl, cold, UV, oxidative stress.



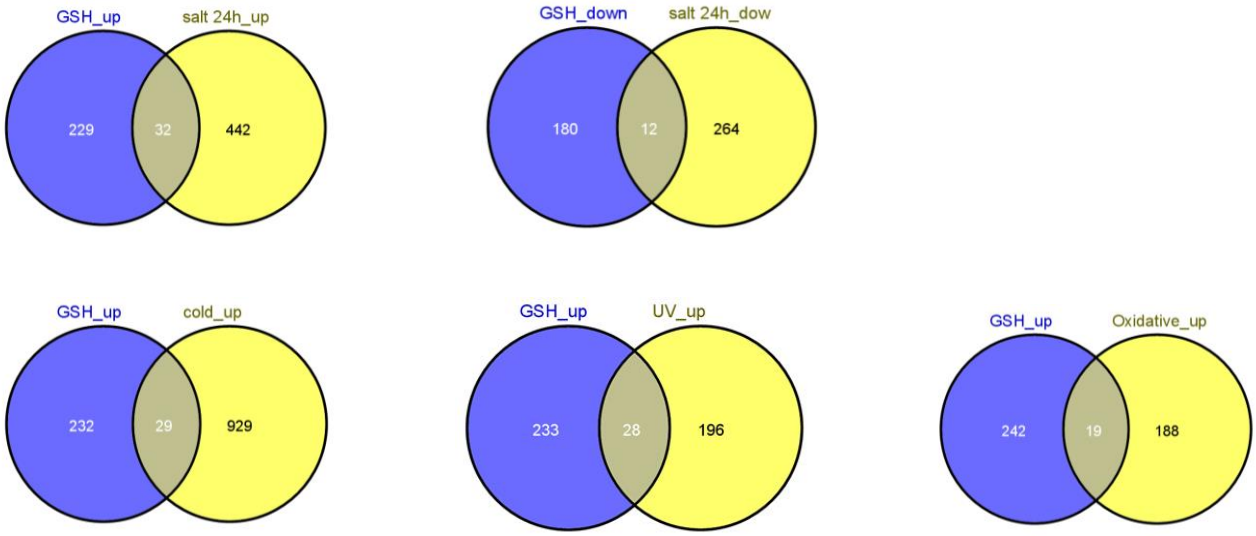


Figure 4. Venn diagrams illustrating the number of genes that were up regulated or down regulated by GSH treatment (blue) and different abiotic stress (yellow). At cold, UV, oxidative stressown regulated genes are not indicated since there were less than 4.

To validate the microarray results, quantitative real time PCR (qPCR) was performed on the same mRNA samples used for the microarray experiments. 4 genes from the 261 up regulated genes were selected for the qPCR analysis. As shown in Fig X all 4 genes showed elevated expression in GSH treatment.

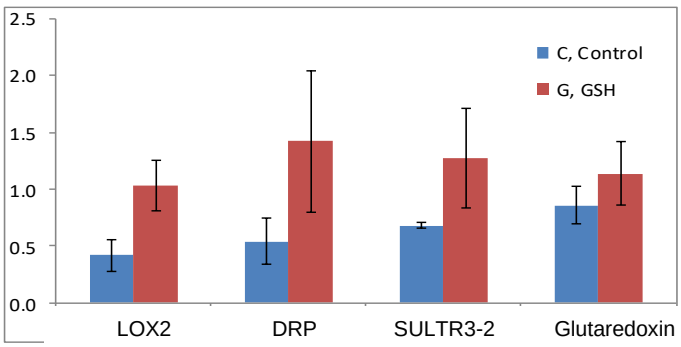


Figure X. Real time PCR analysis for 4 genes that were up regulated by GSH treatment. Lipoxygenase 2 (at3g45140); DRP, Disease resistance protein (at5g41740); SULTR3-2, Sulfate transporter 3;2 (at4g02700); Glutaredoxin family protein (at1g06830).

Among individuals genes that there expression increased were: beta-amylase (BMY5 5 and BMY8), previously it was showed that induction at temperature extremes was specific for two members of the gene family (BMY7 and BMY8) (Plant Physiology, July 2004, Vol. 135, pp. 1674–1684). In addition the activity of TR-BAMY is strongly regulated by the redox potential (Plant Physiol. Vol. 141, 2006). Genes associated to TCA (Carbonic anhydrase 1, Carbonic anhydrase (BCA5)), Cell wall modification genes (xyloglucan, pectinesterase, fucosyltransferase), Redox associated genes (thioredoxin (ACHT2), glutamylcysteinyl-transferase, glutaredoxin), adenosylmethionine decarboxylase, glutathione S transferases, sulphate transports.

Among individuals' genes that there expressions decreased were: methionine gamma-lyase (methionine catabolism), ACC oxidase 1, methionine sulfoxide reductase, myrosinases.

Oliver D. USA-

Study on how altered levels of GSH affect Met and Cys metabolism.

In order to study the impact of GSH on plant metabolism, GSH levels were transiently altered by feeding Arabidopsis wild type plants with GSH or buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis. Liquid cultures were initiated by inoculating 50 surface-sterilized Arabidopsis seeds in liquid B5 media. Feeding treatments were conducted 14 days after inoculation by administrating GSH (2mM), BSO (1mM), or water, as a control, directly into the culture medium. Plants were harvested 6h, 12h and 24h after the beginning of the treatment. Plant tissues were washed twice with deionized H_2O. All liquids on the surface of plant materials were blotted with paper towels. The fresh weight of plant materials was recorded and plants were flash frozen in liquid nitrogen to quench the metabolism.

To investigate how altered level of GSH affects and regulates processes in Met/Cys/GSH metabolism, metabolic profiling of amino acids and derivatives was first done by fractionation and gas chromatography analysis. This targeted approach aims to determine changes in metabolites required for GSH, Cys, or Met synthesis.

Frozen seedlings were ground in liquid nitrogen with a mortar and a pestle and aliquots of the frozen powder (100mg) were extracted in 750 µL of hot methanol for 10 min in a water bath at 60°C. For the quantification of metabolites, 20µl of ribitol (0.2mg/1 mL), 20 µl of nonadecanoic acid (0.2mg/1 mL), and 20 µl of norleucine (0.2mg/1 mL) were added as internal standards. After incubation, the insoluble materials were removed by centrifugation at 21,000 g for 10 min. To separate the polar and non-polar metabolites, the supernatant was carefully mixed with 400 µl of chloroform and 300µl of water. The upper polar phase was taken and reduced to dryness under a nitrogen flow. The pellet was resuspend in 200µl of water for amino acids analysis. The apolar phase was transferred to a new tube, evaporate to dryness under a nitrogen flow and placed at -80°C for further analysis.

Amino acids profiles were determined using the EZ:faast GC-FID kit for free amino acid analysis from Phenomenex (Torrance, CA) according to the instructions provided by the manufacturer. The procedure consists of a solid phase extraction step followed by a derivatization and a liquid/liquid extraction. Derivatized samples were analyzed by gas chromatography with FID detection.

High level of GSH induces strong changes in amino acids profiles (Figure 1). As expected, the level of Met decreases over the 24h of treatment confirming the regulatory role of GSH on Met content. This reduction occurs with an increase of Cys level at 6h. More Cys is available for GSH synthesis but this pool is not used because of the high level of GSH after feeding treatment. The transformation of Cys after 12 and 24h leads to a decrease of its concentration. As the pool of Cys is important after 6h, the level of Ser remains high during the first 12h indicating no transformation for Cys synthesis. The level of Ser drops down after 24h possibly to reconstitute the pool of Cys that might be used for GSH synthesis. The accumulation of Gly over the 24h of treatment can arise from GSH break down. The level of Glu remains unchanged during the 12 first hours and decreases after 24h when GSH synthesis might start over. The level of Gln, His, and Pro are directly linked to the level of Glu (their precursor). During the first 6h of treatment, Glu is temporarily not transformed into Gln and His and their level decrease. After 12 and 24h of treatment, the process is reverse. Glu is transformed into Gln and His.

Among metabolites of aspartate family, the decrease of Asp can be explained by the production of Asn. The increasing level of Thr after 6h of treatment might be a direct consequence of the Met decrease, which leaves more homoserine available for Thr synthesis. The levels of amino acids derived from pyruvate (Leu, Val, Ala) are increased over the time of treatment as well as aromatic amino acids.

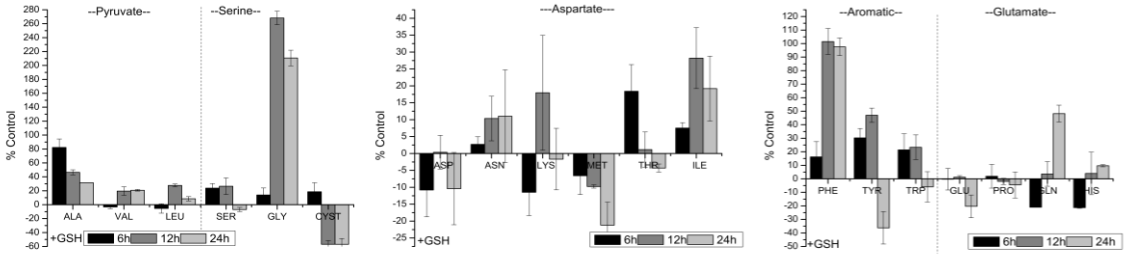


Figure 1: Changes in amino acids levels after 6, 12, 24 h incubation of Arabidopsis seedlings in 2mM GSH. Quantification of amino acids was carried out relative to wild type untreated control plants. Results are the mean ±SE (n=3).

Treatment with BSO solution blocks the synthesis of GSH and shuts down the regulation by GSH which might induce an increase in Met level after 12 and 24h of treatment.

The level of Cys is deeply affected during the first 12h of BSO treatment and Cys might be used to produce Met and Met derivatives (Figure 2). At the same time the levels of Ser is increased, indicating that the pool of Cys is not replaced. After 24h the level of Ser dropped and Cys is accumulated. The level of amino acids (Pro, His , Gln) derived from Glu was increase after 12 and 24 h of BSO treatment. The level of aromatic amino acids is increased during the first 12 h, especially for Tyr.

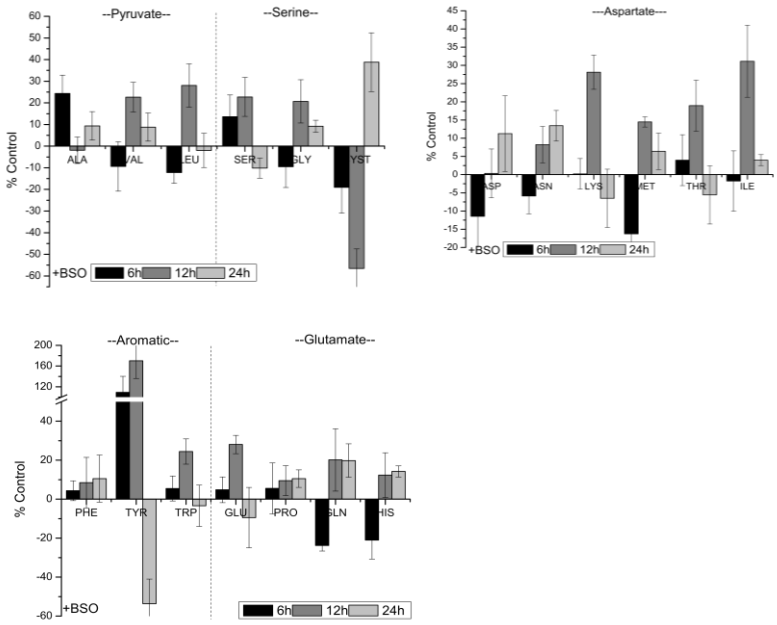


Figure 2: Changes in amino acids levels after 6, 12, 24 h incubation of Arabidopsis seedlings in 1mM BSO. Quantification of amino acids was carried out relative to wild type untreated control plants. Results are the mean ±SE (n=3).

Study how altered levels of GSH/Cys/Met affect the metabolic profiles of plants

To study how high level of Cys and Met can affect the competition for Cys between GSH and Met biosynthesis pathway, the level of Cys and Met were transiently increased using feeding experiments (Figure 3 and 4). Plants were grown in liquid B5 media on a shaker and Cys (1mM) or Met (1mM) were directly administrated into the culture medium. Metabolites extraction was performed on fresh material and metabolic profiling of amino acids and metabolomics analysis were done as described above.

A high level of Met was measured in plants fed with Met. As observed with the BSO feeding experiment, the levels of Cys decreases during the first 12h of treatment showing the use of Cys for *de novo* synthesis. By the same time the levels of Ser is increased, indicating that the pool of Cys is not replace. After 24h the level of Ser drop down and Cys is accumulated. While the level of Glu was not significantly affected, the level of Gln, His and Pro was decreased over the time of feeding with Met. This might indicate that Glu does not produce any Pro, Gln or His and might be channel toward GSH synthesis. Except for glutamate-derived compounds, most of the amino acids (Ala, Val, Leu, Gly Cys, Thr, Ile, Phe, Tyr, Trp) present the same profile. Their concentrations tend to be low after 6h of treatment and increase continuously up to 24h of treatment. No major changes were recorded in the level of Asp, Asn and Lys.

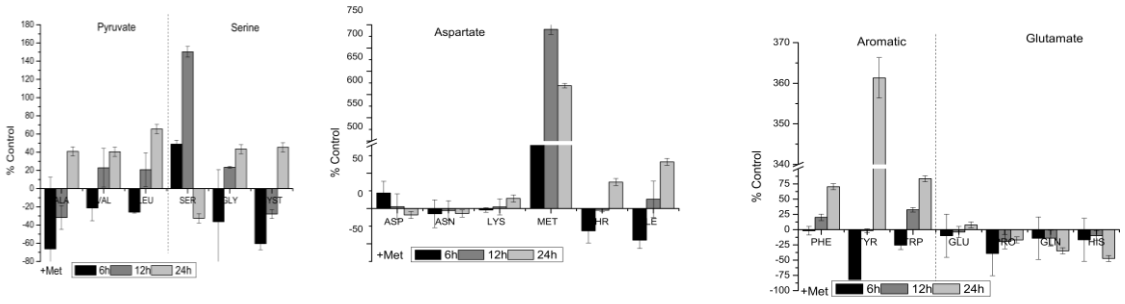


Figure 3: Changes in amino acids levels after 6, 12, or 24 h incubation of Arabidopsis seedlings in 1mM Met. Quantification of amino acids was carried out relative to wild type untreated control plants. Results are the mean ±SE (n=3).

The plants fed with Cys present a high level of Cys while the level of Met first increased after 6h and then decrease after 12 and 24h (Figure 4). Both pyruvate- and aromatic-derived amino acids are accumulated after 24 h of treatment.

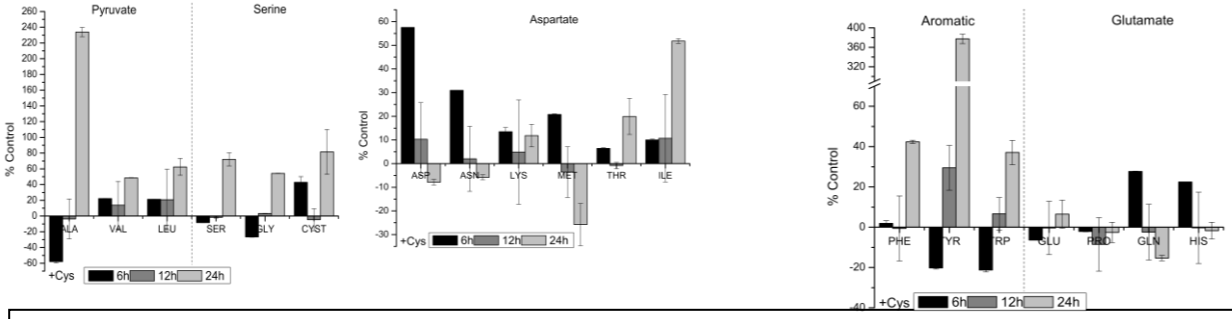


Figure 4: Changes in amino acids levels after 6, 12, 24 h incubation of Arabidopsis seedlings in 1mM Cys. Quantification of amino acids was carried out relative to wild type untreated control plants. Results are the mean ±SE (n=3)

Metabolic profiling and metabolomics analysis of hundreds of compounds using GC/MS were also used to identify metabolites changes that are indirectly connected with altered level of GSH and changes in unknown metabolites. The analysis of the data generated by GC/MS is currently under process.

The role of cysteine partitioning into glutathione and methionine synthesis during normal and stress conditions

To get more knowledge on the competition between Met and GSH on the level of Cys, Arabidopsis plants were grown in MS media for 10 days, and then GSH, Met, Cys, BSO, an inhibitor of ECS that lead to reduction in the GSH content, or paraquat that lead to oxidative stress, were added to the medium for 4 hours. The plants were then collected, frozen in liquid nitrogen, and stored for further analysis. The levels of free amino acids and the levels of thiols were determined using GC-MS and HPLC, respectively. High level of Met in plants treated with Met was found as expected, showing that the amino acids can penetrate the cell membrane and affect the level of metabolites inside the plants. A low level of Ser was found in plants treated with GSH and Met, and a high level of Thr was found in plants treated with GSH. A high level of Thr was found when the level of Met was reduced since the biosynthesis pathways of these two amino acids compete for the same substrate. Ser is precursor for Cys synthesis and thus when high GSH is added, its level was reduced. A low level of Glu was detected in plant treated with Paraquat. High levels of Cys, Cys-Cys, and Tyr were present in plants treated with Cys (Table 8). The results show that GSH and paraquat applications reduced the level of Met, supporting our idea that the elevation of GSH that occurs during oxidative stress and when GSH is applied reduced the level of Met.

Table 8- The levels of soluble amino acids as detected by GC-MS of Arabidopsis plants exposed to different substrates for 4 hours. The levels of amino acids that increased compared to control were marked in green, while those whose levels decreased are marked in red.

	Control	GSH	BSO	Meth	Cyst	Paraquat
ALA	2.49	2.90	2.44	2.39	2.92	2.55
GLY	4.04	4.29	3.07	3.69	4.42	3.12
VAL	1.80	2.63	2.13	2.31	2.30	2.54
LEU	1.03	1.25	0.96	1.11	1.10	1.09
ILE	0.74	0.93	0.75	0.91	0.78	0.75
THR	1.13	1.48	1.17	1.82	1.35	1.45
SER	9.32	4.55	7.61	2.60	8.50	7.97
PRO	94.82	72.74	79.20	73.16	95.20	82.38
ASN	21.57	21.13	21.66	24.27	21.74	23.46
ASP	5.74	5.32	5.93	5.06	5.71	5.43
MET	0.35	0.00	0.15	4.21	0.00	0.00
GLU	7.46	7.37	7.10	7.98	6.75	5.97
PHE	0.39	0.41	0.38	0.44	0.42	0.39
CYS	0.22	0.23	0.23	0.31	1.45	0.28
GLN	58.44	56.50	62.41	66.87	61.03	73.99
LYS	0.34	0.47	0.42	0.45	0.50	0.46
HIS	0.45	0.75	0.78	0.75	0.81	0.64
TYR	0.09	0.13	0.15	0.14	0.26	0.16
TRP	0.07	0.17	0.18	0.10	0.16	0.10
CC	0.06	0.18	0.19	0.10	6.75	0.18

Most of the soluble amino acids are usually incorporated to the proteins. Thus, to determined the total changes in the levels of these amino acids, protein were hydrolyzed and the levels of amino acids were determined by GC-MS. Most of the amino acids content does not alter after 4 hours including Met. The levels of most of them significantly increased in the application of paraquat most probably due to protein degradation. Notably the level of Met did not alter when Cys was applied. Similar results obtained when Met25 gene encoded for the last enzyme of Cys pathway expressed in plants. In these plants the level of Cys and GSH increased but not Met.

Table 9- The levels of total amino acids as detected by GC-MS of Arabidopsis plants exposed to different substrates for 4 hours. The levels of amino acids that their level increased compare to control were marked in green, while those that their levels reduced marked in red.

	Control	GSH	BSO	Meth	Cyst	Paraquat
ALA	1.04	1.24	1.64	0.99	1.61	1.96
GLY	4.78	7.03	6.31	4.16	5.51	7.45
VAL	2.72	2.78	4.09	2.66	3.76	5.01
LEU	3.69	3.76	5.57	3.46	5.07	6.55
ILE	1.36	1.36	2.15	1.11	1.85	2.32
THR	5.30	4.92	6.79	4.71	6.08	10.04
SER	5.85	6.37	5.75	5.49	4.82	13.76
PRO	20.02	18.92	27.69	19.67	28.40	41.02
ASN	0.17	0.21	0.59	0.25	0.21	0.27
ASP	11.17	9.75	13.59	8.94	11.76	20.47
MET	0.43	0.32	0.64	1.19	0.42	0.43
GLU	13.79	12.85	14.49	11.05	14.22	26.63
PHE	1.75	1.65	2.47	1.53	2.22	2.85
GLN	1.51	0.99	2.92	0.77	1.63	2.09
orn	1.17	1.47	2.29	1.53	1.95	3.82
LYS	2.97	3.16	4.84	2.99	5.03	6.27
HIS	1.66	1.56	2.42	1.68	2.16	3.02
TYR	0.89	0.87	1.56	1.29	1.65	2.04
TRP	0.04	0.04	0.04	0.04	0.04	0.04
CC	0.21	0.27	0.48	0.50	0.80	0.99

The levels of thiols was also measured after 4 hours using HPLC (Fig. 4). The level of GSH significantly increased when GSH is applied, showing that GSH was taken up by the plants. Also plants supplied with Cys shows higher GSH content as expected. In these plants the level of Met does not increased. Application of GSH and Cys increased the levels of Cys as expected. GSH is known to degrade to Cys and this explains the elevation of Cys in these plants. The levels of homocysteine, one metabolite up-stream from Met, increased when GSH is applied, suggesting that Met synthesis by the last enzyme does not operate when GSH is found at high concentrations.

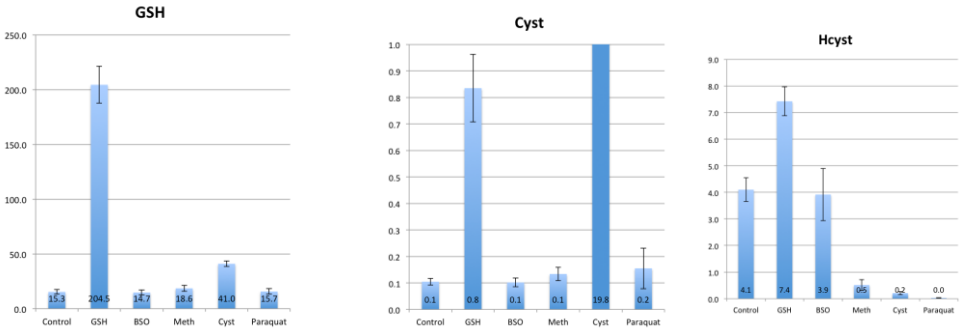


Figure 4- the levels of GSH, Cys and homocysteine in the Arabidopsis plants supplied with different compounds for 4 hour.

The levels of thiols were also measured after 8 hours from the application (Fig. 5). The results were similar to those described in Fig. 4, obtained for GSH and Cys content. The main differences were found in the level of homocysteine.

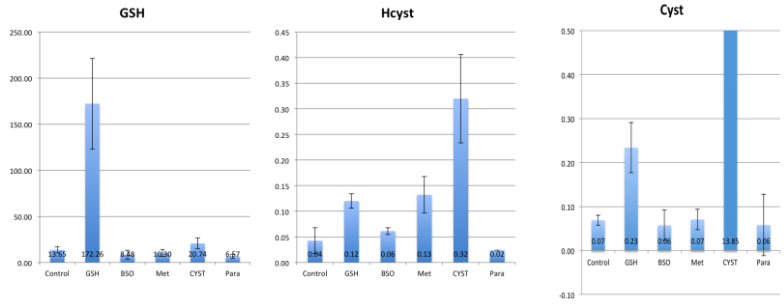


Figure 5- the levels of GSH, Cys and homocysteine in the Arabidopsis plants applied with different compounds for 8 hour.

Next, we measured the levels of soluble and total amino acids after 8 hours of application. The level of Met decreased when GSH was applied (Table 10). Application of

BSO that reduced the level of GSH increased the level of Met. Total amino acids were measured (Table 11) and no differences were observed except that the level of Met increased when Met applied.

Table 10- The levels of soluble amino acids as detected by GC-MS of Arabidopsis plants exposed to different substrates for 8 hours. The levels of amino acids whose levels increased compare to control were marked in green, while those whose levels decreased marked in red.

Average	Control	GSH	BSO	Meth	Cyst	Paraquat
ALA	0.67	0.69	0.87	0.73	1.21	0.71
GLY	3.14	1.88	9.84	4.88	5.78	1.43
VAL	0.69	0.51	0.56	1.12	1.12	1.82
LEU	0.35	0.22	0.23	0.39	0.74	1.43
ILE	0.18	0.11	0.13	0.22	0.39	0.82
THR	3.07	3.28	3.53	3.07	4.31	5.10
SER	17.83	17.98	31.25	20.86	17.14	8.87
PRO	15.86	10.51	11.65	14.58	16.70	12.59
ASN	10.13	10.18	13.31	11.38	9.13	10.81
ASP	8.58	6.28	10.46	6.45	6.87	5.87
MET	0.10	0.06	0.12	27.55	0.30	0.05
GLU	5.84	10.13	8.95	10.60	8.92	1.38
PHE	0.35	0.40	0.42	0.65	0.87	0.58
CYS	0.08	0.05	0.09	0.16	0.64	0.07
GLN	65.27	68.34	134.35	97.24	73.41	98.49
LYS	0.18	0.27	0.37	0.22	0.49	1.01
HIS	0.46	0.65	0.75	0.74	0.71	0.65
TYR	0.09	0.24	0.21	0.15	0.48	0.45
TRP	0.06	0.15	0.14	0.11	0.27	0.13
CC	0.00	0.26	0.00	0.00	2.22	0.00

Table 11- The levels of total amino acids as detected by GC-MS of Arabidopsis plants exposed to different substrates for 4 hours. The levels of amino acids whose levels increased compare to control were marked in green, while those whose levels decreased are marked in red.

	Control	GSH	BSO	Meth	Cyst	Paraquat
ALA	1.83	1.34	1.16	1.22	0.90	1.21
GLY	8.76	8.63	6.89	7.14	6.24	6.28
VAL	6.49	4.67	4.42	4.88	3.77	4.77
LEU	12.79	9.54	9.04	9.86	9.00	9.05
ILE	6.27	4.60	4.31	4.86	4.21	4.64
THR	5.75	4.87	4.50	5.66	4.21	3.71
SER	3.88	2.80	3.47	4.12	3.62	6.00
PRO	9.56	6.39	5.60	7.58	6.03	6.74
ASN	1.02	0.56	1.02	1.36	1.07	0.90
ASP	10.96	8.48	7.56	8.80	7.29	8.84
MET	0.82	0.88	0.83	3.81	0.78	0.64
GLU	15.26	13.13	11.08	12.41	10.28	15.03
PHE	5.47	4.50	4.27	4.50	4.64	4.09
GLN	2.82	1.27	1.44	1.64	1.32	3.54
orn	0.23	0.28	0.24	0.25	0.09	0.21
LYS	2.54	2.08	2.13	1.89	1.83	2.70
HIS	1.84	2.10	1.90	1.95	1.63	1.47
TYR	2.45	2.18	1.91	1.84	1.87	2.04
TRP	0.02	0.02	0.02	0.02	0.02	0.02
CC	0.53	0.57	0.47	0.51	0.45	0.44

Basically, the results shown in this section are in accordance with our hypothesis, suggesting that GSH application reduced the level of Met synthesis. Slightly lower levels of GSH obtained when BSO was applied increased the levels of Met. The levels of other amino acids also behave as expected, while other not and further thinking need to be put to reveal this issue.

Metabolic flux measurements

Metabolic flux measurements were undertaken using a modification of the method of (Baxter et al. Plant Physiol. 143:312-325 [2007]). The basic idea is to feed [U-^{13}C]-glucose to small Arabidopsis plants and then use a combination of GC-MS and HPLC-MS to measure the flux of label into the amino acids, organic acids, and other molecules of interest. We initially had difficulties getting sufficient labeling into the amino acids because of the amount of carbon coming from the ^{12}C-sucrose the plants were grown on. The following method was devised to circumvent this limitation. Four-week-old Arabidopsis seedlings (n=6-7) grown on 0.1% sucrose with half strength MS media were transferred to Whatman No. 1 filter papers which were wetted with 0.2% glucose with half-strength MS media. After 2 days, 2 ml of 2% [U-^{13}C]-glucose (with or without paraquat) in MS media was added. The seedlings were harvested at 6, 12, 24, and 48 hours into liquid nitrogen. The plants were freeze-dried for about 48 hours before powdering them using Geno/Grinder. About 10 mg of dry material was extracted with methanol/chloroform/water and the aqueous phase dried under vacuum. The residue was derivatized with *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) (Figure 1) and analyzed by GC-MS. Metabolites were identified by comparing to retention times and unique mass fragment patterns of standards from NIST mass library. In each case the largest or intact derivatized ion was determined for further analysis.

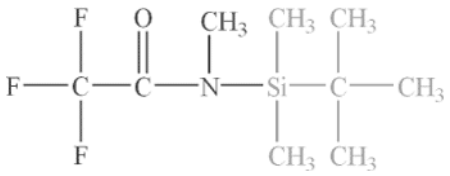


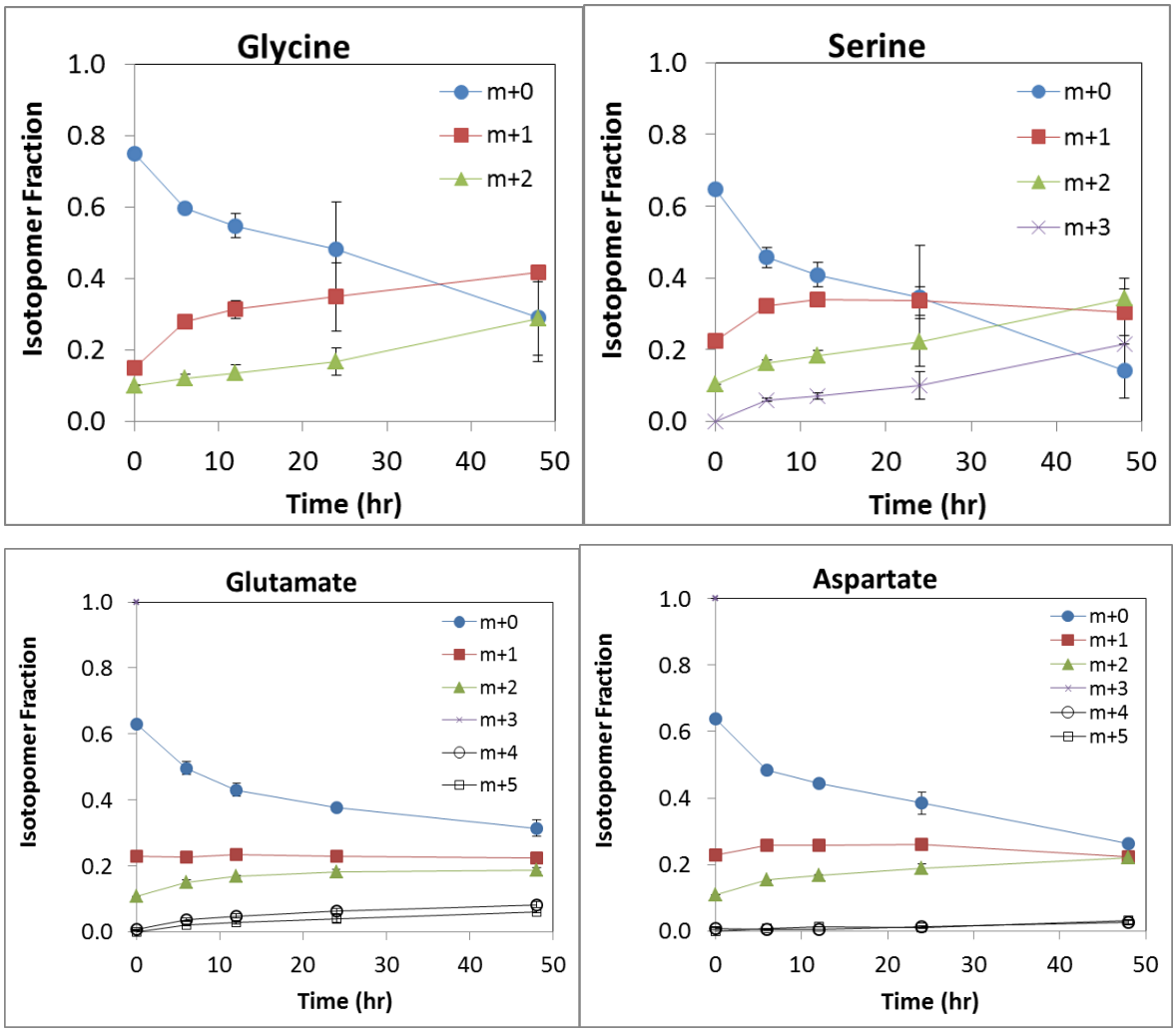
Figure 6. Molecular structure of *tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). The portion of the chemical in gray color (tert-butyldimethylsilyl, TBDMS) derivatizes to carboxyl and amino functional groups.

The basic idea in analyzing these results is that the preexisting compounds are built out of ^{12}C while the newly made molecules contain ^{13}C. There is a natural abundance of heavy isotopes that are in the original compound but these are ignored from this description and blanked arithmetically during the actual analysis. In Figure 2 the preexisting molecules with only ^{12}C are labeled m+0 (mass plus zero). The incorporation of one ^{13}C results in m+1, the incorporation of two ^{13}C in m+2, and so forth. The decrease in m+0 measures the rate of metabolism of the compound and the increase in m+1, m+2, m+3, etc. measure the rate of synthesis of the compound. The data in Figure 2 shows that serine and glycine turnover (both metabolism and synthesis) was faster than the other amino acids illustrated. The rates of flux

through the glutamate and aspartate were moderate while the rates of flux for the branched amino acids, valine and leucine, were very low. These values can be used to compare the flux rates for different metabolites as well as to look for changes in flux rate under different conditions.

The levels of most amino acids and metabolites in TCA cycle increased or did not change while those of citrate, alanine, and glutamine decreased over time (Figure 3). While the method can measure methionine levels, during this experiment insufficient materials were analyzed in the GC-MS to get measurements of the m+1 and m+2 ions to get quantitative data. We have shown that we can get the data needed by doing parallel GC-MS analyses on larger samples and this modification will be incorporated into the follow up experiment.

We are at present in the middle of a large fully replicated experiment that will determine flux rates for methionine as well as the rest of the amino acids and most of the organic acids in the Krebs cycle. The ^{13}C-glucose feeding will take place next week. It will talk about a month to do the sample processing, derivatization, GC-MS, and data analysis.



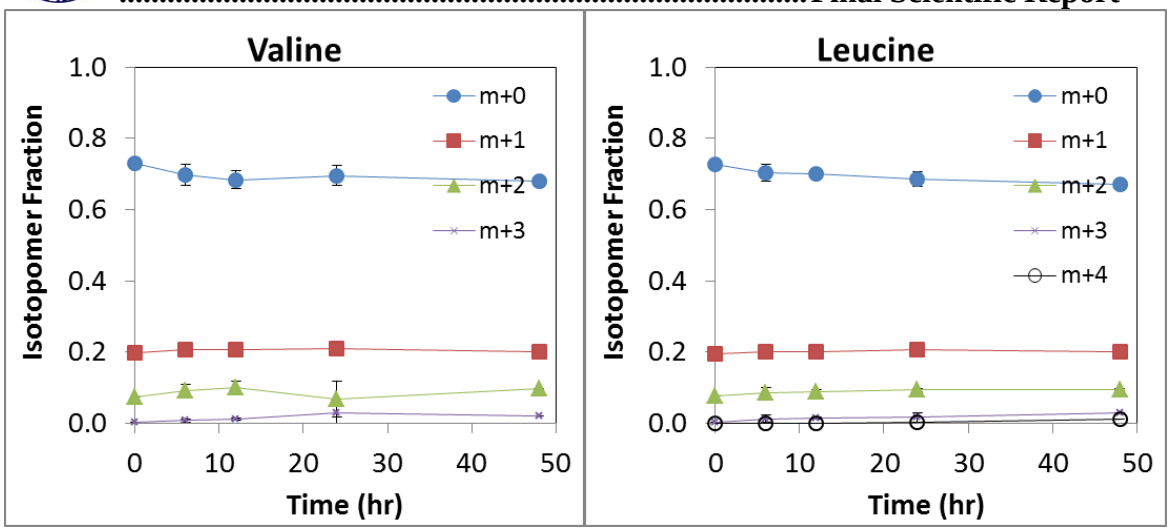
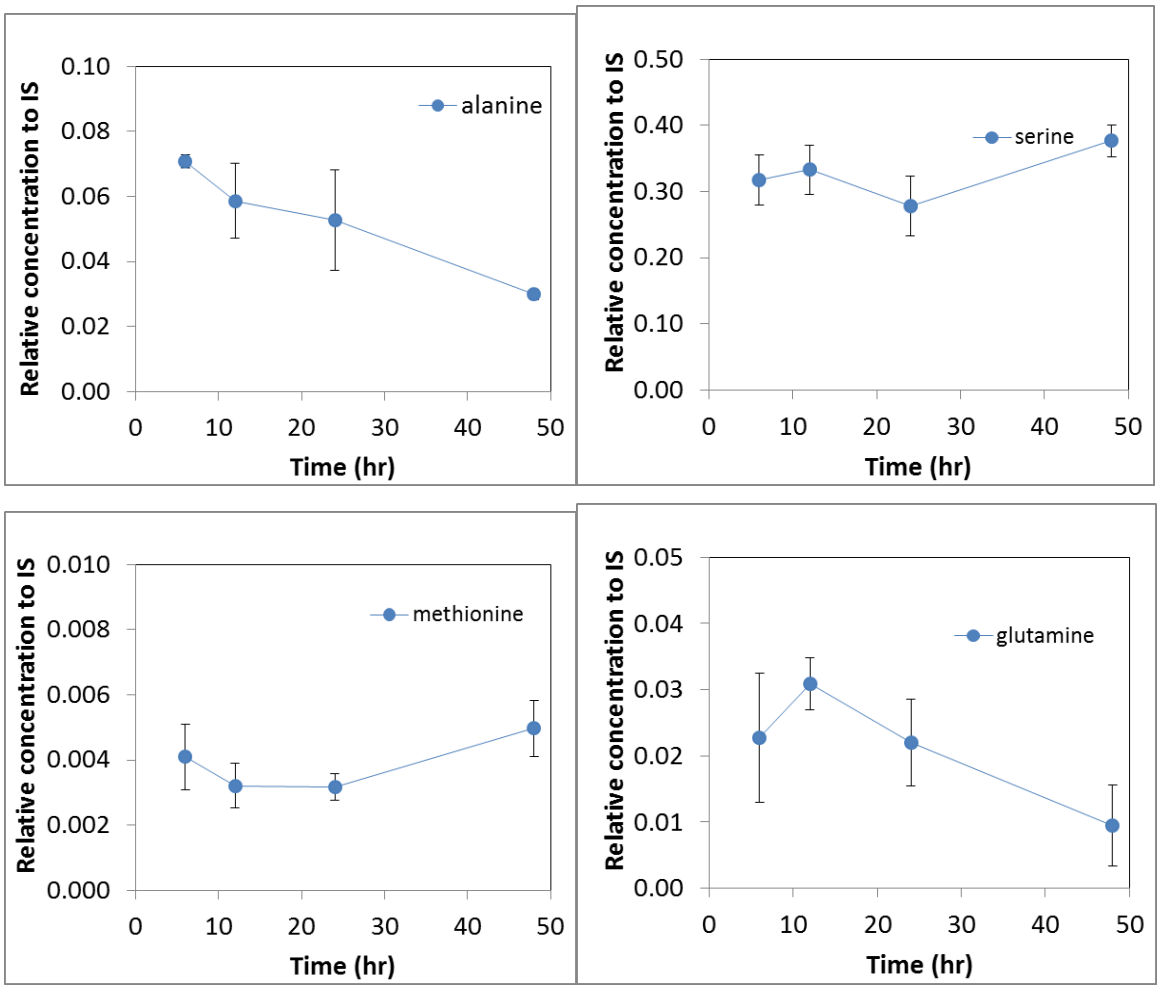


Figure 7. Time course for synthesis and metabolism of several selected amino acids in Arabidopsis plants. As the ^{13}C labeled-carbon replaces the natural ^{12}C- carbon the fraction of m+0 decreases while the fraction of m+1, m+2, etc increases. The rate of m+0 decrease is proportional to the rate of metabolism of this compound while the rate of formation of the heavier molecules is proportional to the rate of its biosynthesis. Flux rates and their changes can be calculated from these values.



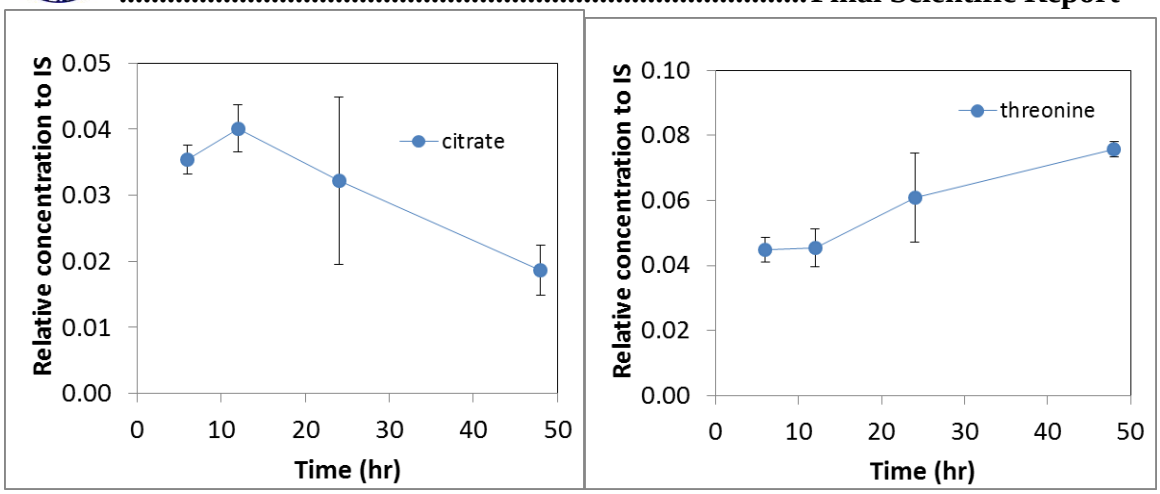


Figure 8. Relative concentration of selected metabolites during the course of these ^{13}C-glucose feeding experiments.

Now that we have demonstrated that we can measure the rate of flux through these different compounds additional experiments are underway that will provide rates of flux through Met thus allowing us to verify the hypothesis that GSH and oxidative stress control the partitioning of Cys into Met.

Conclusions:

The data presented strongly support our initial hypothesis that GSH and Met synthesis compete for available Cys. Stress conditions cause the flow of sulfur from Cys into GSH and these elevated GSH levels repress the synthesis of Met. Mechanistically this regulation seems to result from a post-translational control of CGS (cystathionine γ-synthase). Current work is focused on final proof that the changes in Met levels result from the rate of Met synthesis and details of the mechanism by which CGS is regulated.

Metabolomics and Metabolic Flux Experiments.

The purpose of the metabolomics and metabolic flux experiments is to verify that oxidative stress caused by either hydrogen peroxide (H_2O_2) or methyl viologen (MV) treatments resulted in a decrease in methionine and an increase in glutathione levels and then to determine if this resulted from a decrease in the rate of methionine biosynthesis. These experiments were done with both Arabidopsis and tobacco seedlings. Seedlings were germinated on solid MS media on square petri dishes that were held vertically. After two (Arabidopsis) or four (tobacco) weeks the seedlings were removed from the media and transferred to plates containing dampened filter paper (Figure 1). At time 0 the plants were provided with ^{13}C-glucose (for the flux measurement) with or without 3 mM methyl viologen

or the indicated concentration of H_2O_2. At the times indicated, up to 22 hours, the plants were harvested to measure methionine concentration, glutathione concentration, and the amount of ^{12}C and ^{13}C in methionine.

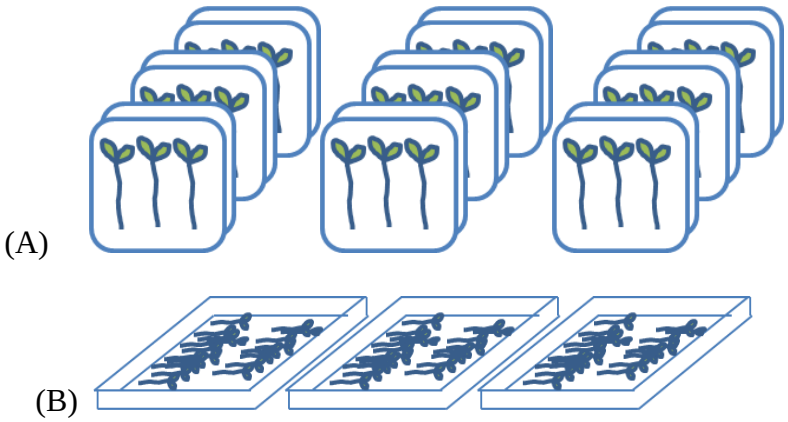


Figure 1. Illustration of the growth conditions for the metabolic flux experiments. A - The plants were initially grown on MS media containing 2% sucrose. B - In order to measure the metabolism of ^{13}C-glucose and to feed either H_2O_2 or methyl-viologen, the plants were removed to filter papers.

Figure 2 shows the effect of hydrogen peroxide concentration during a 22 hour treatment with tobacco seedlings. The data shows total glutathione (GSH and GSSG) and reduced glutathione (GSH). The amount of oxidized glutathione (GSSG) is the difference between the two. The H_2O_2 treatments caused the oxidation of the glutathione pool and a greater than two fold increase in the total glutathione level reflecting increased glutathione biosynthesis. Full flux analyses are currently being done on these seedlings and those results will be available soon.

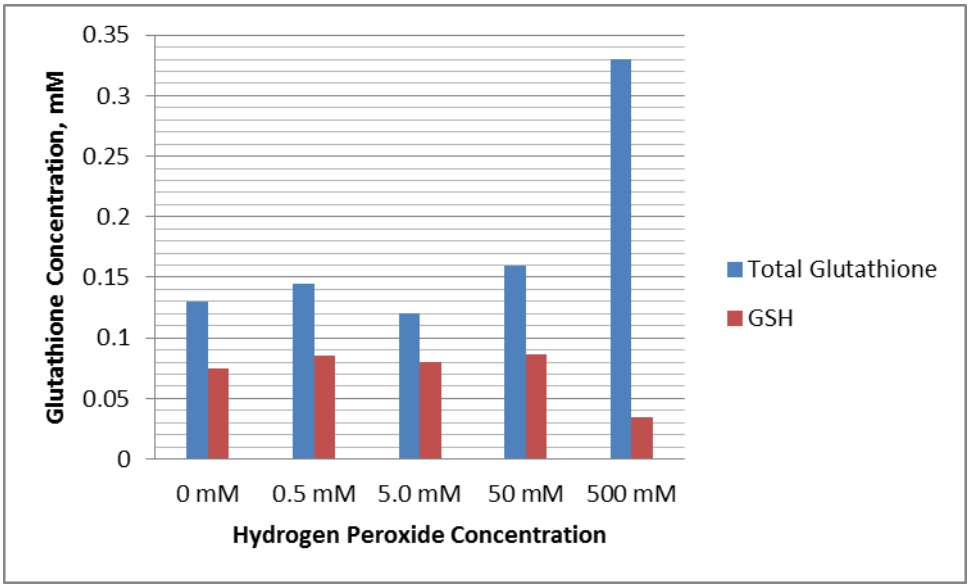


Figure 2. The concentraiton of reduced GSH and total (GSH plus GSSG) in tobacco seedlings treated with H_2O_2

Methyl viologen (MV), like H_2O_2, also caused oxidative stress. The stress results in oxidation of reduced GSH to GSSG. In Figure 3 Arabidopsis plants were treated with 0 mM or 3 mM MV for up to 12 hours. During the treatment period the GSH level was largely unchanged in the control plants (0 mM MV) while the reduced GSH was converted to oxidized GSSG in those plants exposed to 3 mM MV. This resulted in a near total loss of GSH but not change in total glutathione (GSH and GSSH) (Figure 3). We are not sure why tobacco and Arabidopsis are responding differently to oxidative stress.

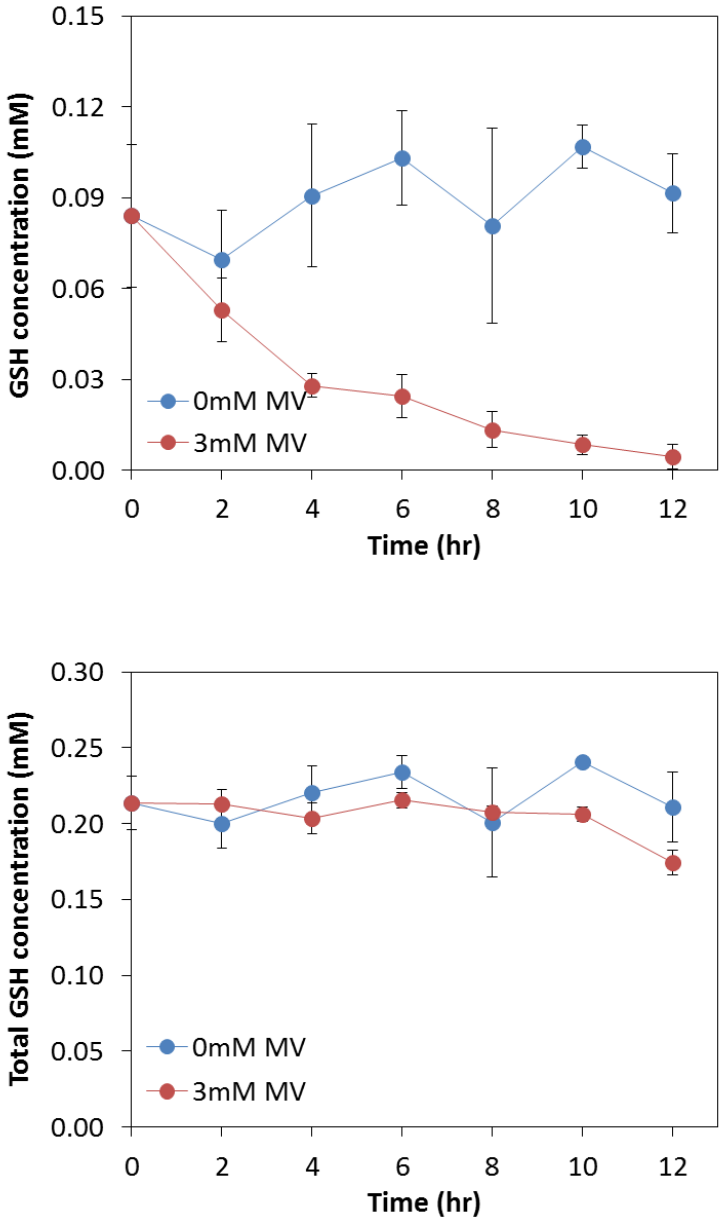


Figure 3. Concentration of reduced GSH (top) or total glutathione (bottom) in Arabidopsis plants on water (blue line) or 3 mM MV (red line).

As predicted from the earlier results, as cysteine is channeled into the synthesis of glutathione, the level of methionine decreases. This was confirmed in the methyl viologen-treated Arabidopsis plants (Figure 4). During the 12 hours of the experiment the methionine level rose in the control plants (0 mM MV) but dropped in the MV-treated plants (3 mM MV). The decrease in the MV level averaged about 30% through the experiment.

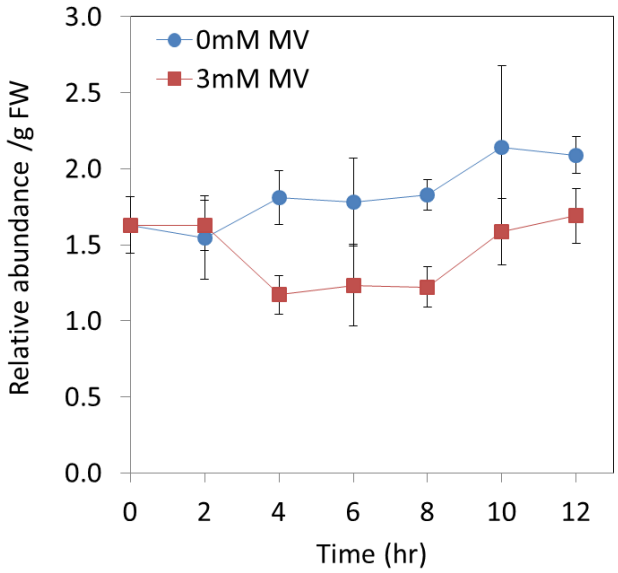


Figure 4. Methionine level during the 12 hour experiment in control plants (blue) and plants treated with 3 mM methyl viologen. The concentrations are in relative units off the mass spectrometer.

In order to determine if this drop in methionine level following MV treatment resulted from a decrease in the rate of methionine synthesis, metabolic flux experiments were undertaken. Briefly the plants are treated with 0 mM or 3 mM MV and exposed to ^{13}C-glucose. At 2 hour intervals plants are harvested and the methionine isolated and analyzed by mass spectrometry for the amount of ^{13}C it contains. The rate at which the methionine pool accumulates ^{13}C and loses ^{12}C is a measure of the flux of carbon from ^{13}C-glucose into methionine – the rate of methionine synthesis.

For plants that were not treated with MV, Figure 5(top) shows the fraction of methionine molecules that have no ^{13}C (the mass plus zero or M + 0 value), the fraction of methionine that has one ^{13}C (M + 1), the fraction with two ^{13}C (M + 2), etc. At time 0, the fraction of M + 0 is about 0.73 because of the natural abundance of ^{12}C and ^{13}C in the atmosphere. During the course of the experiment the fraction of M + 0 methionine decreases and the others increase as ^{13}C flows from glucose into methionine. The slope of that curve is

proportional to the rate of methionine synthesis. During the time of the experiment the M + 0 fraction drops from 0.73 to 0.58.

The results for plants treated with 3 mM MV were substantially different (Figure 5 bottom). In the MV-treated plants the M + 0 line only decreases slightly during the 12 hours (from 0.73 to 0.71). Thus the rate of methionine synthesis from ^{13}C glucose is much lower in MV-treated plants than in control plants.

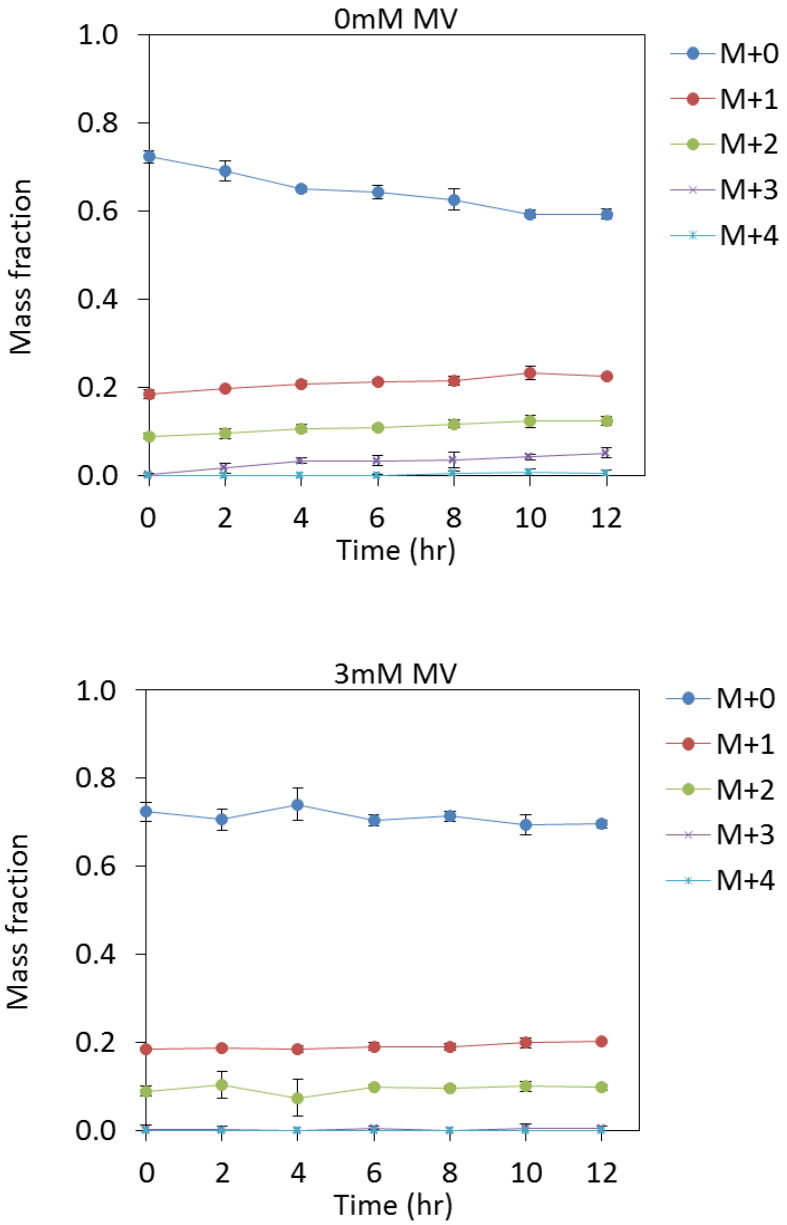


Figure 5A. The fraction of the methionine pool that contains no ^{13}C atoms (M + 0), one ^{13}C atom (M + 1), two ^{13}C atoms (M + 2), three ^{13}C atoms (M + 3), and four ^{13}C atoms (M + 4). The ^{13}C-glucose was introduced at time 0 and plants were sampled for analysis at the times indicated. Plants were exposed to 0 mM (top) or 3 mM (bottom) methyl viologen.

Figure 6 gives direct comparison of the rates of change in the fraction of ^{13}C in the methionine pools in control and MV-treated plants. This is the same data in Figure 5 put into one figure to illustrate the difference in slope of the two lines. The rate of methionine synthesis is directly proportional to the slope of the lines. The MV treatment decreased the rate of methionine synthesis by 80% to 90%. These results conclusively show that the treatment with MV causes a large decrease in the rate of methionine synthesis.

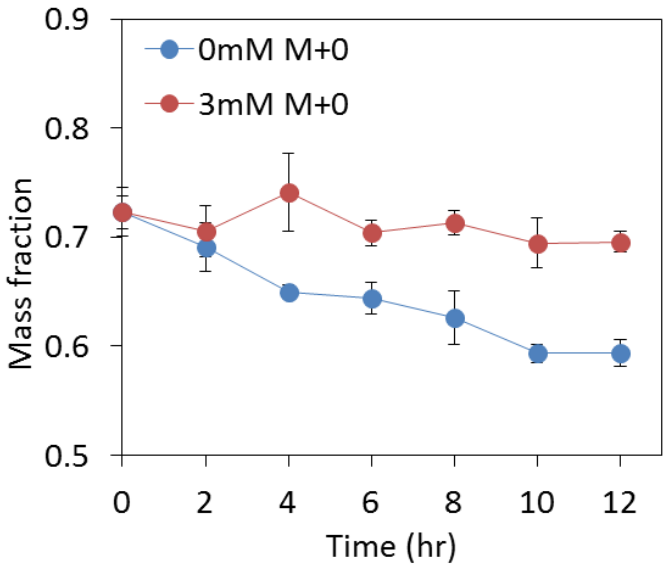


Figure 6. The rate of methionine synthesis from ^{13}C-glucose in control and MV-treated plants. These data are the M + 0 lines from Figures 5A (0 mM MV) and 5B (3 mM MV) where the slopes of the line are proportional to the rate of flux into methionine in the absence (blue line) or presence (red line) of methyl viologen.

Here is the total glutathione (GSH and GSSG) in wild type plants treated with or without 150 mM H_2O_2. Total glutathione levels increased with hydrogen peroxide treatments (Figure 7).

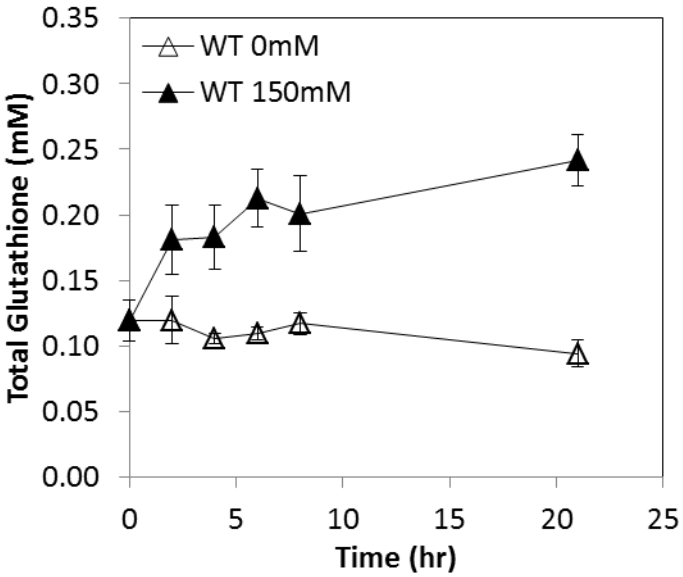


Figure 7. Total Glutathione Levels in Wild type ± 150 mM H_2O_2

The level of reduced glutathione (GSH) levels in plant treated with or without H_2O_2 are shown in Figure 8. The GSH levels drops with hydrogen peroxide treatment. These results are exactly as expected. The oxidative stress causes by hydrogen peroxide treatment converts GSH into GSSG and the plants respond by making more glutathione.

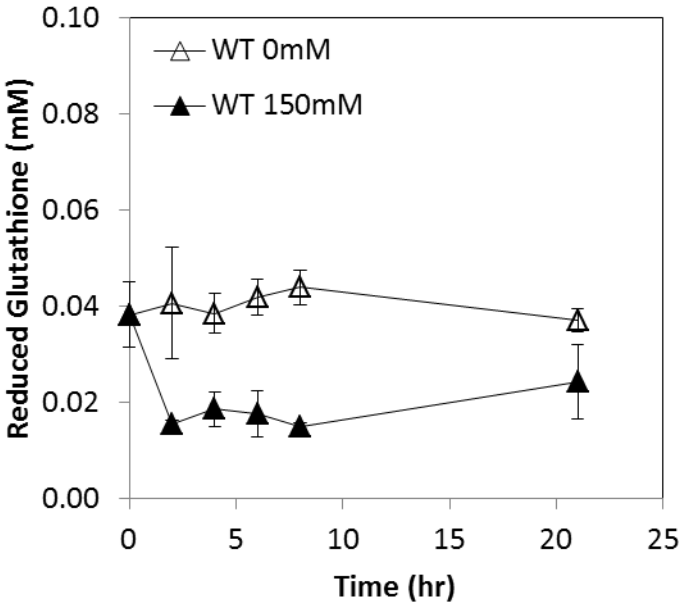


Figure 8. Reduced GSH Levels in Wild type ± 150 mM H_2O_2

The methionine levels are shown in Figure 8. The methionine levels are very constant during the 20 hours of the experiment in the controls (0 mM H_2O_2). There is no significant difference between the control and the treated (150 mM H_2O_2) for the first 8 hours of treatment. There is a 30% drop in the MET levels of the treated tissues at 20 hours. These

results are reasonably consistent with what we saw before with Arabidopsis but in those earlier experiments the decrease in methionine occurred earlier.

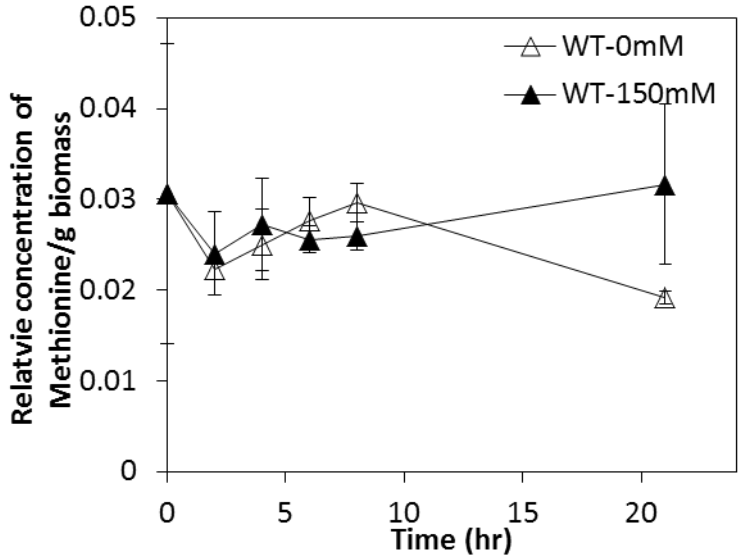


Figure 9. Methionine Levels in Wild type ± 150 mM H_2O_2

The flux data for the wildtype plants treated with 0 mM or 150 mM H_2O_2 are shown in Figures 10 and 11. Remember what we are looking for is the rate of M+0 decrease and the rate of increase of M+1 & M+2 & M+3 & M+4 & M+5 as ^{13}C from ^{13}C-glucose is converted into methionine.

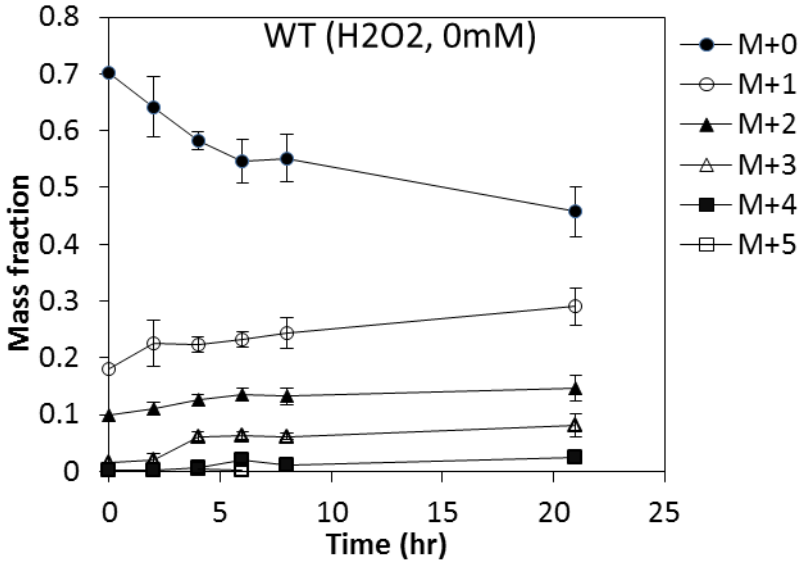


Figure 10. Mass Fraction in Wild type 0 mM H_2O_2

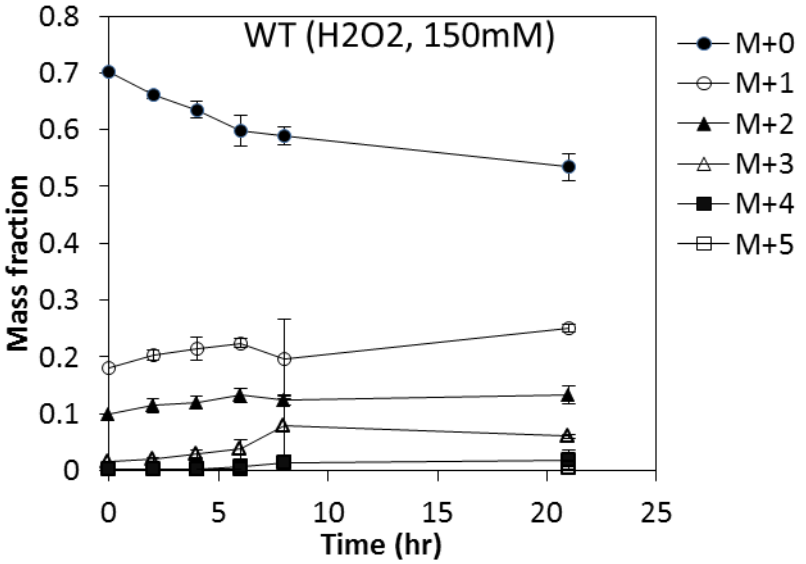


Figure 11. Mass Fraction in Wild type + 150 mM H_2O_2

This is easier to see if we just look at only the M+0 (methionine that has no 13C) data (Figure 12). The slope of the curve in the decrease in mass fraction of M+0 is proportional to the rate of methionine synthesis. We can make this assumption because the methionine concentration does not change much with time. The results are clear. When the plants were treated with H_2O_2, the rate of methionine synthesis decreased. In this experiment the rate was about 36% less than in the untreated plants. Clearly hydrogen peroxide treatment decreases the rate of methionine synthesis with wild type tobacco.

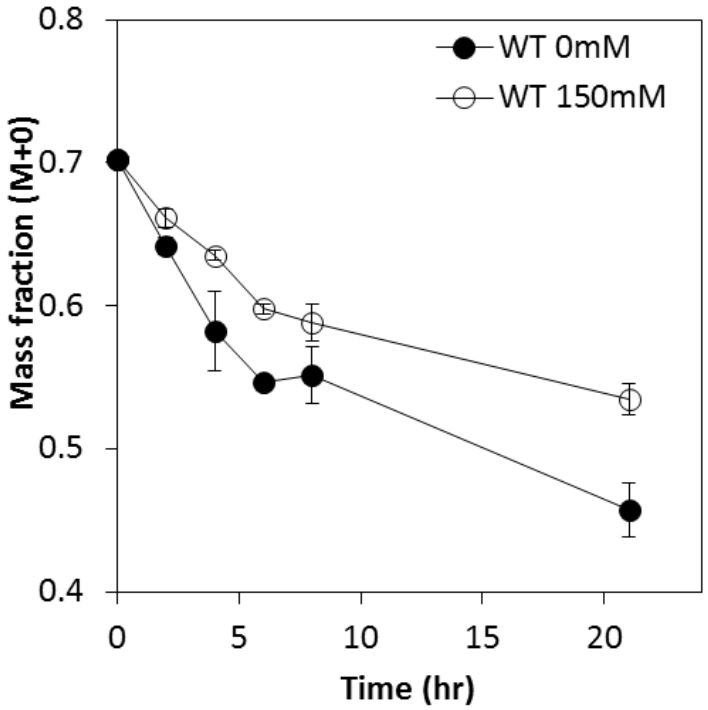


Figure 12. Comparison of Mass Fraction in Wild type ± 150 mM H_2O_2

The FCGS line

The FCGS plants respond like the wildtype in terms of total glutathione. As with the wildtype the treatment with hydrogen peroxide causes an increase in the amount of total glutathione (Figure 13). The initial glutathione level and the change in glutathione level is about the same for wild type and FCGS plants (compare Figures 7 and 13).

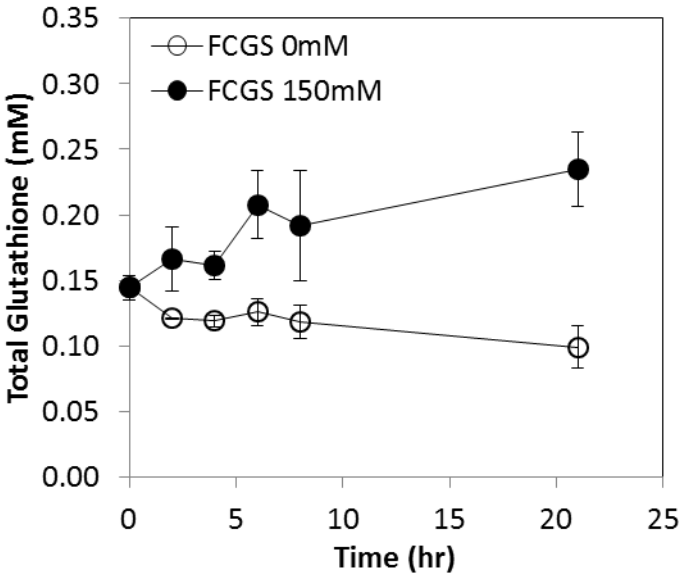


Figure 13. Total Glutathione in FCGS ± 150 mM H_2O_2

There were some differences in how reduced GSH levels changed in wild type and FCGS plants following hydrogen peroxide treatment. While in both cases there was a rapid decrease in GSH levels, in the FCGS plants the GSH levels recovered to the level of untreated plants (Figure 14). In wild type plants recovery was slower and not to the same extent (Figure 8).

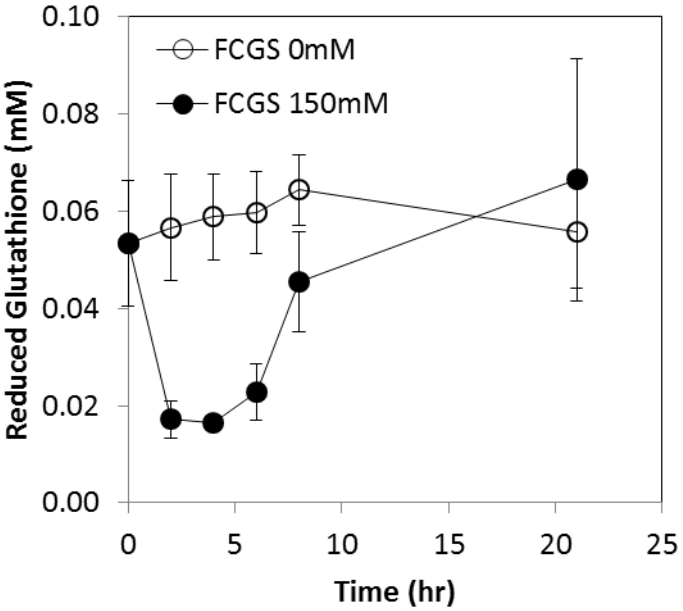


Figure 14. Reduced GSH in FCGS ± 150 mM H_2O_2

We had problems with the methionine levels in the FCGS plants. The variability between plants was large and as a result the data have very large error bars. The error bars are so large that it is impossible to draw many conclusions. One conclusion that we can draw is that the FCGS plants have about 10-times more methionine than the wildtype plants. That is very consistent and statistically significant even with our error bars. What we can not show is that hydrogen peroxide treatment has any effect on methionine levels in FCGS plants. If hydrogen peroxide treatment is decreasing methionine levels in FCGS plants then that difference is lost in the error measurements.

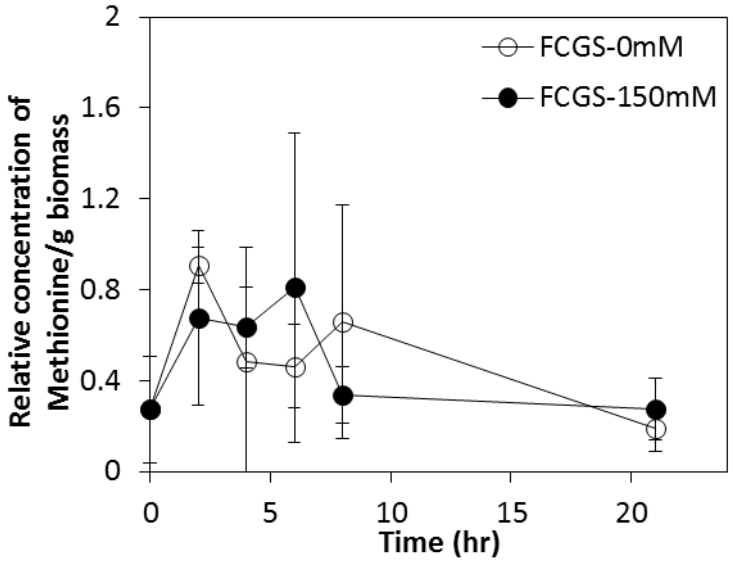


Figure 15. Methionine Level in FCGS ± 150 mM H_2O_2

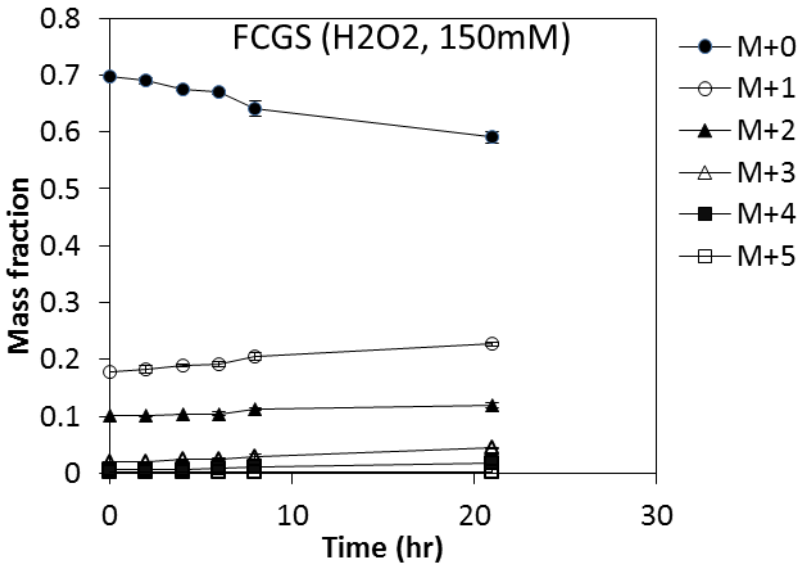


Figure 16. Mass Fraction of FCGS + 150 mM H_2O_2

Again if we just take the M+0 data it is easier to compare. Figure 12 has the M+0 data for the FCGS plants. There are two points that need to be taken away from this.

1. Comparing the rate of methionine synthesis between the wildtype and the FCGS line. The slope of the line is determined by the size of the methionine pool and the rate that the pool is turned over. So while the slope of the line for FCGS (Figure 17) is less than for wildtype (Figure 12) the methionine pool in the wildtype is much smaller (Figure 9 and 15). If for example half of the wildtype pool turned over in a day that is a lower rate of methionine synthesis than a quarter turnover of the much larger pool in FCGS in the same period. So the slope for the wildtype change is 2.3 times larger than for FCGS but the FCGS methionine pool is 10-times larger than for wildtype. If you put these two points together you end up that methionine is synthesized 2 to 3-times faster in FCGS than in wildtype.

2. Comparing the effect of H_2O_2 on the rate of methionine synthesis in FCGS. Looking at just the FCGS data in Figure 17 there is no difference between the rate of methionine synthesis in the presence or absence of hydrogen peroxide. I am not sure why this would be true. Results like this would result if the CGS transgene was not controlled by oxidative stress while the endogenous gene in the wildtype plants was controlled by oxidative stress.

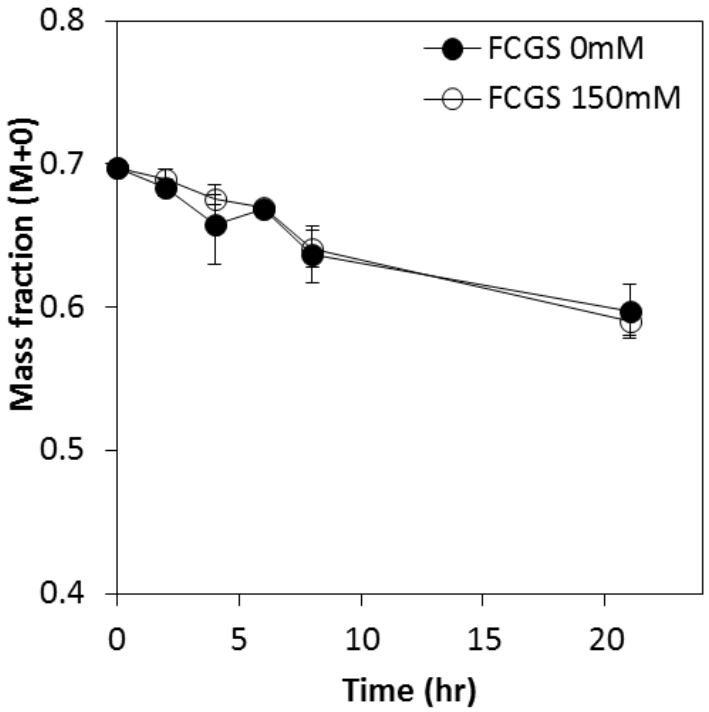


Figure 17. Comparison of Mass Fraction in FCGS ± 150 mM H_2O_2